No. 82

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American Journal of

Veterinary RESEARCH

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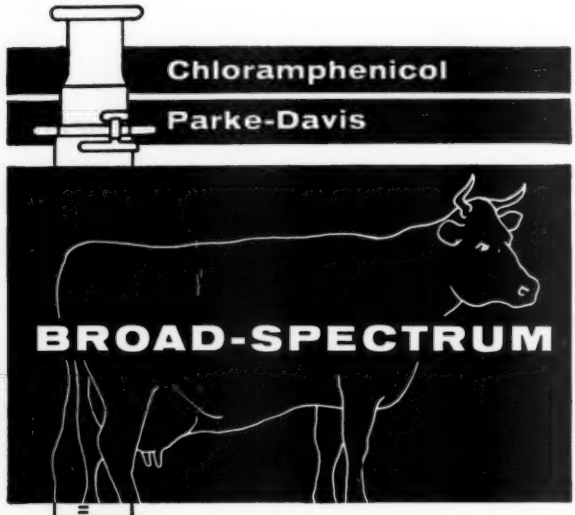

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A Journal of the British Veterinary Association
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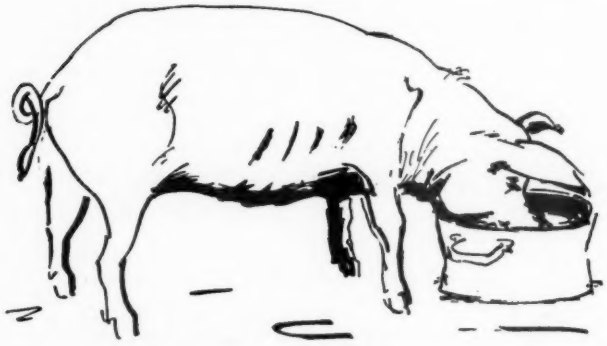

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Survey of Cardiovascular Disease in Dogs—Preliminary Report on the First 1,000 Dogs Screened

D. K. DETWEILER, V.M.D., M.S.; K. HUBBEN, V.M.D., M.S.; D. F. PATTERSON, D.V.M.

Philadelphia, Pennsylvania

THE PURPOSE of this study was to determine the prevalence and types of spontaneous cardiovascular disease occurring in dogs of all ages presented to the veterinary clinic and hospital of the University of Pennsylvania. Previous studies at this institution [9-19] indicated that the prevalence in dogs of diseases of the heart and blood vessels was considerably greater than generally suspected.

LITERATURE

That canine cardiovascular disease has, until recently, received little attention is shown by the relatively meager information available in the older literature. In 1925, Ackerknecht [1] reviewed the existing literature on cardiac disease in domestic animals, including the dog and, together with Krause,[2] reviewed the literature on diseases of the blood vessels in 1929. Since that time, no comprehensive reviews of the literature have appeared, although a number of reports dealing with various types of heart disease have been published, especially during the last decade. Because most of these papers have been referred to in previous publications,[9-19] only a few will be cited here.

In 1949, Lannek [27] published a detailed statistical apalysis of the normal electrocardiogram of the dog, together with observations on a series of animals showing electrocardiographic abnormalities. This work served as the cornerstone of clinical canine electrocardiography in the following years. The first of the series of publications from this institution on heart disease in dogs appeared in 1952.[9] This work summarized existing knowledge and reported the results of several years of clinical study. In 1957, Dahme [6] reviewed the literature on arterial disease in dogs and reported original studies which emphasized the types of vascular disease found in association with chronic interstitial nephritis. A chapter summarizing the clinical aspects of cardiovascular disease in dogs has appeared recently.[19]

METHODS

Electrocardiography.—Electrocardiograms were taken with either a Cambridge Versa-Scribe (heated-stylus, direct-writing), Edin electronic cardiograph (ink-writing), or a Sanborn twin-beam photographic two-channel recorder. Alligator clips attached to the lead cables were used as electrodes. These were clipped directly to the skin to which conventional electrocardiographic electrode paste was applied.

For screening purposes, one of the direct-writing electrocardiographs was employed and a "semi-unipolar" chest lead, CR_{6L}, was registered with the dog in the standing position. The indifferent electrode was connected to the right foreleg, and the exploring electrode to the bottom of the left sixth intercostal space. When multilead electrocardiograms were taken, the subject was placed in lateral recumbency on its right side. Routinely, in dogs suspected of having heart disease, the following leads were taken: standard limb leads I, II, III; augmented unipolar leads, aVR, aVL, aVF, and three precordial leads (established by Lannek [27]) against Wilson's central terminal (V). The electrode positions for the chest leads are labelled CV_{6U}, CV_{6L}, and CV_5 in accordance with Lannek's terminology.[27] The exploring electrode is located at the sixth left intercostal space at the costochondral junction for lead CV_{6U}; at the bottom (near the edge of the sternum) of the sixth left intercostal space for lead CV_{6L}; and at the fifth right intercostal space near the edge of the sternum for lead CV_5. In the normal dog, the exploring electrode in lead CV_{6U} is over the free wall of the

From the School of Veterinary Medicine, University of Pennsylvania, Philadelphia.

Presented before the Section on Small Animals, Ninety-Sixth Annual Meeting, American Veterinary Medical Association, Kansas City, Mo., Aug. 23–26, 1959.

This work is supported by Research Grant H-3541 from the National Heart Institute of the National Institutes of Health.

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left ventricle, in CV_{6L} it is near the left ventricular apex, and in CV_5 over the right ventricle.

Phonocardiography and Heart Sound Recording.—A Sanborn twin-beam two-channel recorder was used for recording phonocardiograms and a Cambridge audio-visual heart sound recorder was employed for making magnetic disk recordings of heart sounds and murmurs.

Auscultation.—The established auscultation areas of the heart (mitral, tricuspid, aortic, and pulmonic valve areas [19]) were auscultated, using a binaural stethoscope. Abnormalities in timing, duration, location, pitch, and intensity of the heart sounds and any murmurs were recorded. Murmurs, when present, were graded as to intensity in accordance with the following arbitrary classification: grade 1, the faintest audible murmur; grade 2, faint murmur, clearly heard after a few seconds auscultation; grade 3, murmur which is immediately audible when auscultation begins and heard over a fairly large area (a thrill accompanies the more intense); grade 4, the loudest murmur which is still inaudible when the stethoscope is just removed from the thoracic wall (a thrill is always present); grade 5, extremely loud murmur which can still be heard with the stethoscope just removed from the thoracic wall (a thrill is always present).

Palpation of the Precordium.—The animal is approached from the rear and one hand is placed on each side of the thorax over the heart. Normally, the cardiac impulse can be felt most strongly against the left hand at about the fifth, left intercostal space. Variations indicating a change in position of the heart, the presence of thrills, or abnormal arrhythmias were recorded. Lateral curvature of the spine or leaning of the animal toward the right or left can affect the location of the heart relative to the thoracic wall. Therefore, care was taken during palpation to maintain the vertebral column straight and to keep the weight distributed equally on the four feet.

Palpation of the Femoral Arteries.—The femoral arteries were palpated simultaneously, comparing the relative character of the pulse in each, and noticing an absent or relatively diminished pulse in one or both.

Blood Pressure Determination.—Systolic blood pressure was determined by detecting the earliest appearance of a pulse wave below an inflatable cuff. For this purpose, the arterial pulse was detected by a sensitive capacitance microphone (Brecht-Boucke microphone). The signal, suitably amplified, was recorded with an electrocardiograph. The pressure cuff was connected through tubing with an Infraton Automatic Pressure Marker [20, 22] (Medical Electronics Development Co.). This consists of an aneroid manometer for reading the pressure and a mercury manometer arranged with a system of electrical contacts so that signals are fed into the electrocardiograph at given pressures. Thus, both pulse waves and pressure marks are recorded. With the dog lying on its side, the microphone is strapped to the paw and the cuff is wrapped about the leg above the tarsal joint of one hindleg. The cuff is inflated until no pulse signal is recorded from the microphone. As the cuff is deflated, marks are automatically recorded at given pressures. The point at which the arterial pulse first appears in the tracing is taken as the systolic arterial blood pressure (fig. 1).

In some cases, blood pressure was determined by direct puncture of the femoral artery or with the auscultatory or palpatory method using a Riva Rocci cuff applied to the hindleg above the tarsus and palpating or ausculting the dorsal pedal artery.

Standards have not been established for these methods under our conditions. Animals showing excessive systolic pressures (e.g., exceeding the 190 mm. Hg with the capacitance-microphone method) when first examined are re-examined and, if possible, trained to see if the pressure decreases when excitement is minimized.

Roentgenography.—Thoracic roentgenograms were taken at a target-film distance of 36 inches. Routinely, a dorsoventral view and a lateral view were obtained. For both, the x-ray tube was centered over the heart. For lateral views, the animals were placed on their left side. Oblique views were occasionally employed.

The cardiac silhouette was studied carefully for evidence of distortion of any of the borders or for evidence of enlargement of any of the chambers or the whole heart.

Lateral thoracic roentgenograms have been found most useful in determining enlargement of

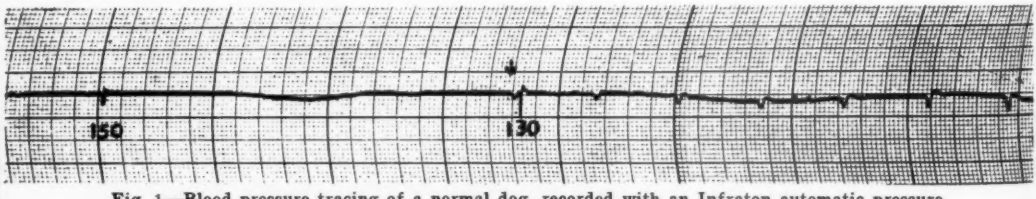

Fig. 1—Blood pressure tracing of a normal dog, recorded with an Infraton automatic pressure marker. The arterial pulse was recorded by a sensitive capacitance microphone strapped to an extremity. An inflatable cuff above the microphone was used to occlude the arterial circulation. As the cuff was deflated, pressure marks were automatically recorded at given pressures. The point at which the arterial pulse was first recorded was taken as the systolic arterial blood pressure. In this case, the systolic pressure was approximately 130 mm. of Hg.

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the heart as a whole or disproportionate enlargement of one or more chambers. In the lateral view, the anterior border of the cardiac silhouette is formed by the right ventricle and atrium; the posterior border, by the left ventricle and atrium. Both the anterior and posterior borders curve inward at the base of the heart and then become continuous with the vascular shadows of the anterior and posterior mediastinum. Thus, the silhouette of the base of the heart narrows in the region of the atrioventricular groove and widens where it becomes confluent with the venae cavae and pulmonary veins, producing a waistlike appearance in the region of the atria. When the heart is enlarged as a whole, both anterior and posterior borders are straightened, causing this "waist" near the base of the heart to become less pronounced or to disappear. When the right side of the heart is enlarged, this straightening of the anterior border causes the angle formed by the vascular shadows of the anterior mediastinum and the anterior heart border to become less acute. Similarly, when the left side of the heart is enlarged, the angle formed by the pulmonary veins as they enter the left atrium and the posterior heart border becomes more obtuse. Thus when either the right or left side of the heart is enlarged primarily, the anterior or posterior aspect, respectively, of the waist is lost.

The trachea and mainstem bronchi over the base of the heart are deviated dorsally as the dorsoventral diameter of the heart increases. The angle formed near the thoracic inlet by the long axes of the thoracic spine and the trachea becomes more acute. In cases of extreme cardiac enlargement, the tracheal shadow appears to be in contact with the thoracic spine shadow.

When the right atrium is disproportionately enlarged, the trachea is bowed upward anterior to the mainstem bronchi and often one mainstem bronchus (right) is elevated so that the end-on views of the two bronchi are seen to be separate rather than confluent. When the left atrium is disproportionately enlarged, the left mainstem bronchus is elevated and often the atrial shadow can be seen between the two air-filled bronchi, which now appear as a V-shaped structure with one branch above the other (*see* fig. 17).

The pulmonary arteries were identified and examined for evidence of enlargement.

The lung fields were studied and any indication of congestion, pulmonary edema, neoplastic growth, or other lung disease was noted.

Fluoroscopy was not used routinely. However, it was occasionally employed in cases where pericarditis with effusion or vascular ring lesions incarcerating the esophagus and trachea were suspected, or in any other conditions in which it was desirable to observe the motion of the heart or watch the progress of a barium sulfate solution or bolus as it traversed the esophagus.

Angiocardiography.—Most dogs requiring angiocardiography were taken to the catheterization laboratory of the hospital of the University of Pennsylvania.* The apparatus used was a Westinghouse Cine-Fluorex with 5-inch screen.

The dogs were anesthetized with pentobarbital sodium intravenously. Intracardiac catheters were inserted into either the jugular or femoral vein and passed into the right heart under fluoroscopic control. Pressure records and electrocardiograms were monitored on the cathode ray oscilloscope of a Cambridge multichannel research recorder, and photographic records of the traces made when appropriate.

For angiocardiograms and observations requiring entrance into the left ventricle, we have punctured the free wall of the left ventricle directly with a 20- to 22-gauge needle..

The radiopaque contrast medium employed was Hypaque-M** 90 per cent given in a dose of 0.5 ml. per pound of body weight by rapid injection with a Gidlund high pressure injector (Elema, Stockholm).

Occasionally, angiocardiograms have been made without special apparatus.[31] An 18-gauge needle was inserted into the jugular vein. The radiopaque medium was injected rapidly and two or three roentgenograms were taken as quickly as possible after injection. The film cassettes were stacked under the dog and as each uppermost film was exposed, it was removed.

Clinical Laboratory Work.—The methods for hemograms, hemoglobin determinations, hematocrits, sedimentation rates, urinalyses, and blood chemical determinations were standard. Microfilaria were detected by direct microscopic examination of blood smears and by a concentration method in which 1 ml. of blood is mixed with 10 ml. of 2 per cent formalin solution. This mixture is centrifuged at 1,500 r.p.m. for five minutes. The sediment is mixed with an equal volume of 1:1,000 methylene blue and examined microscopically under a cover slip. Microfilaria were distinguished by employing the criteria recommended by Newton and Wright.[32]

Screening Procedure.—To obtain a representative sample, all dogs entering the small animal clinic each day during regular clinic hours were examined, with the exception of the occasional animal requiring immediate emergency treatment. The few dogs entering the hospital at other hours were screened when cardiovascular disease was suspected or when a cardiac examination was requested by the medicine or surgery staffs.

The number of dogs entering the clinic each day required a brief screening procedure, limited in most instances to three to five minutes for each subject. To avoid interference with the operation of the regular clinic, dogs were brought to the heart station while waiting to be examined. When positive screening results indicated a need for further study, appointments were made for return of

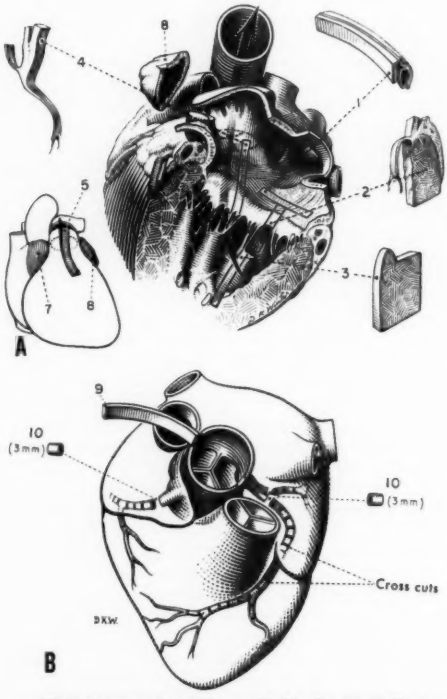
* We are indebted to Drs. Harry Woske, John Helwig, Jr., and Donald F. Hyman, and to Miss Barbara Hummell for carrying out these studies.

** Diatrizoate, Winthrop Laboratories, Inc., New York, N.Y.

the animals to the heart station at a later date, at which time a complete history was obtained and the necessary confirmatory studies made.

The initial screening examination consisted of the following: (1) auscultation of the mitral, tricuspid, aortic, and pulmonic valve areas; (2) palpation of the precordium; (3) palpation of the femoral arteries; and (4) registration of a lead CR_{6L} (see electrocardiography) electrocardiogram.

Classification of Dogs on Basis of Screening Results.—Each animal screened was classified according to one of the following categories: (1) No heart disease: This category included all dogs in which no evidence of heart disease was discovered and in which no conditions known to cause heart disease were present. (2) Heart disease: When dependable signs of heart disease were observed, dogs were placed in this category. (3) Possible heart disease: Animals with signs suggesting cardiac involvement but not presenting dependable signs of heart disease were placed in this category. (4) Potential heart disease: Animals in which no signs of heart disease were present, but which had disease conditions (e.g., heartworm, severe anemia, hypertension) capable of producing heart disease, were placed in this category.



—*AFIP 58166 and 58162, provided by Dr. W. C. Manion*

Fig. 2 (A)—Drawing, showing sites where routine sections should be obtained for histopathologic examination. These sites are selected even in the absence of grossly visible lesions. Additional sections of lesions, when present, should be made.

B) Drawing, showing method for examination of coronary arteries and selection of sections for histopathologic study. Transverse cuts are made every 4 to 5 mm.

Dependable Signs of Heart Disease.—The signs accepted as reliable evidence of heart disease were: (1) a grade 4 or greater systolic murmur; (2) a grade 3 systolic murmur in the absence of anemia (anemic murmurs have not been present at hemoglobin levels above 6 Gm./100 ml. of blood, although the accepted normal range for dogs in our clinic is between 12 and 16 Gm./100 ml.); (3) a diastolic murmur; (4) a palpable precordial thrill; (5) generalized venous engorgement; (6) atrial fibrillation or flutter; (7) paroxysmal ventricular tachycardia; (8) atrial or ventricular extrasystoles consistently present and of frequent occurrence; (9) complete atrioventricular block; (10) left bundle branch block; (11) electrocardiographic right ventricular enlargement pattern; (12) radiographic evidence of gross heart enlargement, enlargement of one or more chambers, or pericardial effusion.

When one or more of the foregoing signs were present, the dog was placed in the heart disease category and further confirmatory studies carried out at a later time.

Signs Suggestive of Heart Disease.—Dogs with the following signs were suspected of having cardiovascular disease and examined further: (1) a grade I or grade II systolic murmur; (2) ascites with or without limb edema; (3) radiographic evidence of borderline cardiac enlargement; (4) incomplete heart block; (5) electrocardiographic RS-T segment shifts of 0.2 mv. or greater; (6) markedly diminished or absent pulsation in one or both femoral arteries.

In some cases, the presence of two or more signs suggestive of heart disease, when considered together, was taken as confirmation of cardiac involvement. An example of this was the finding of a grade II systolic murmur at the mitral valve area in the absence of anemia, accompanied by roentgenograms indicating borderline left ventricular and left atrial enlargement.

In cases showing dyspnea, cough, and ease of fatigability or weakness, an attempt was made to find the underlying cause of these signs so as to rule out incipient heart disease.

If palpation of the precordium indicated cardiac enlargement or displacement, roentgenograms were taken to aid in establishing the cause.

When the arterial blood pressure was elevated (*see* Blood Pressure Determination), the animals were further examined for evidence of left ventricular enlargement, renal disease, or other abnormalities.

Postmortem Examination.—Necropsy procedure as outlined by Coffin was followed.[7] For the most part, dogs in which heart disease was diagnosed antemortem were necropsied by one of the authors

(K.H.). Other dogs were necropsied by members of the Department of Pathology or veterinary students working under the supervision of an experienced pathologist.

Following gross postmortem examination, a descriptive protocol was written and, where appropriate, photographs were taken of lesions. The coronary arteries were examined by transverse incisions every 4 to 5 mm. and by visual examination of the exposed lumens. For histopathologic examination of the heart, tissue blocks were selected as recommended by the Armed Forces Institute of Pathology (fig. 2).

When cardiovascular disease was diagnosed antemortem, the following tissues were taken for histopathologic examination: heart, aorta, brain, spinal cord, lung, stomach, small intestine, colon, pancreas, liver, thyroid, parathyroid, adrenal gland, kidney, bladder, and genital organs. In cases not diagnosed as heart disease, the tissues taken for diagnostic work depended on the postmortem findings.

Tissues were processed in the usual manner and the sections stained with hematoxylin and eosin. During the period covered by this report, few special stains were employed. In subsequent work, connective tissue, fat, elastic tissue, and other special stains have been used.

Samples for microbiologic examination were not taken routinely.

RESULTS

CLINICAL EXAMINATION

During a 16-week period, 1,000 dogs were subjected to the screening procedures and classified in the four categories defined under methods.

The results of the initial screening procedure in 1,000 dogs, and the final distribution in the four categories after clinical confirming studies and postmortem examinations had been completed, are shown (table 1).

Of 828 dogs in which no evidence of cardiovascular disease was found by means of the screening procedure, 18 were necropsied and 9 found to have gross heart lesions. Two dogs which had been classified as III, possible heart disease, after screening had no heart lesions on gross or microscopic postmortem examinations. One dog classified as III, possible heart disease, because of a cardiorespiratory murmur, was reclassified as I after clinical confirming studies revealed no evidence of cardiovascular disease.

After screening, 76 dogs were classified as II, heart disease. Of these, 16 were not confirmed by further study and were reclassified as III. Sixty were confirmed as II by clinical or postmortem examination, or both, and remained in category II. Originally classified as III, 28 dogs were confirmed as having heart disease by clinical (14) and postmortem (14) examinations, and placed in category II. In addition, the 9 dogs which were negative at screening but had heart lesions on postmortem examination were placed in category II, giving a total of 97 dogs with definite clinical or postmortem evidence of heart disease.

Following screening examinations, 95 dogs were placed in category III, possible heart disease. As stated previously, 28 were confirmed as having definite heart disease (II). Because of lack of confirmatory evidence, 16 dogs which were originally considered as being in category II, were transferred to III. Two dogs considered as III after screening were necropsied and found to have no heart lesions. One originally classed as III was subsequently found to have no clinical evidence of cardiovascular disease and was placed in category I with the 2 found negative on necropsy, leaving 80 dogs which had signs suggestive of cardiovascular disease but in which definite evidence was not established. Of these, 54

TABLE 1—Results of Screening 1,000 Dogs for Cardiovascular Disease and Final Distribution After Clinical Confirming Studies and Necropsy Examinations

	Category	Screen result	Final distribution
I	No heart disease	828	822
II	Heart disease	76	97
III	Possible heart disease	95	80
IV	Potential heart disease	1	1

TABLE 2—Distribution of Positive Findings for Each Screening Procedure

Procedure	No. of positive findings		
Auscultation	122		
Murmurs		112	
Others		10	
Palpation	16		
Thrill		12	
Heart displacement or enlargement		4	
Femoral artery palpation	0		
CRsL electrocardiogram	43		
Arrhythmias		7	
RS-T segment shifts		11	
Right heart enlargement pattern		9	
Low amplitude		7	
Conduction delays		9	
Left bundle branch block			2
Right bundle branch block			1
Incomplete heart block			4
Incomplete heart block with dropped beats			2
Total	181		

TABLE 3—Frequency of Positive Clinical Confirming Studies and Postmortem Examinations in Dogs Ultimately Placed in Category II (Heart Disease)

Study	Frequency of significant findings in 1,000 dogs
Radiography	44
Electrocardiography	40
Hematology (to establish absence of anemia)	25
Postmortem examination	49
Total	158

failed to return for clinical confirming studies.

One animal was placed in category IV, potential heart disease, because of a systolic blood pressure (Infraton) of over 220 mm. Hg. Further study revealed no other evidence of cardiovascular disease.

The number of times an abnormality was detected with each screening procedure were tabulated (table 2), together with the nature of the aberration.

Auscultatory abnormalities, found in 122 dogs, occurred most frequently. In 112, murmurs were attributable to valvular lesions or congenital malformations, while other abnormalities (unidentified friction sounds, decreased intensity of both heart sounds) were recorded in 10.

TABLE 4—Clinical Diagnoses in 48 Dogs Not Necropsied

Clinical diagnoses	No. of cases
Pulmonic stenosis (congenital)	1
Chronic atrioventricular valvular disease	27
Chronic atrioventricular valvular and myocardial disease	11
Myocardial disease	2
Cor pulmonale	4
Undiagnosed	3
Total	48

Palpation of the precordium revealed abnormalities in 16 dogs. In 12, thrills accompanied intense murmurs and in 4 there was evidence of cardiac displacement or gross enlargement.

Palpation of the femoral arteries did not add to the positive findings in the screening program. One dog with subacute bacterial endocarditis developed femoral artery thrombosis terminally.

The semi-unipolar precordial lead electrocardiogram revealed abnormalities in 43 dogs. Of the 7 with abnormal arrhythmias, 6 had ventricular extrasystoles and 1 atrial fibrillation. RS-T segment shifts occurred in 11 dogs. RS-T segment depression of between 0.2 to 0.3 mv. in precordial leads was observed in 4 dogs. The depression exceeded 0.3 mv. in 5, and 2 had RS-T segment elevations of between 0.2 and 0.3 mv. The first 4 mentioned are probably within normal limits. None of the dogs were classified as having heart disease (category II) because of RS-T segment changes alone.

Among the 172 dogs placed in categories II, III, or IV after screening, a total of 181 abnormalities were detected during the screening procedure. Thus in only a few instances was more than a single abnormality detected. The placing of 1 dog in category IV was based upon a blood pressure determination. This was not a regular part of the screening program, although resorted to in certain instances when there was time and the subject was considered amenable to the procedure.

The relative importance of individual confirming studies in the final diagnostic classification can be seen (table 3). In many cases, several studies of the same animal revealed reliable evidence of cardiac disease.

The distribution of clinical diagnoses in the 48 dogs which had definite signs of heart disease on clinical examination, but were not necropsied is shown (table 4). The most common diagnosis was chronic valvular disease, while the combination of chronic valvular and myocardial disease was the next most common diagnosis. Definite electrocardiographic signs of myocardial disease without evidence of valvular disease were observed in 2 dogs. Among 4 dogs with evidence of cor pulmonale, *Dirofilaria immitis* microfilaria were observed in the blood of 1. In one 6-month-old dog, a clinical diagnosis of congenital pulmonic stenosis was made on the basis of roentgenographic and electrocardiographic evidence of right ventricular hypertrophy and the presence of a crescendo-decrescendo systolic murmur in the pulmonic area. This dog was asymptomatic at the time of examination. Three dogs had definite signs of heart disease, but complete diagnoses were not made because of insufficient clinical study.

POSTMORTEM EXAMINATION

The necropsy material included in this report is divided into two groups: (1) dogs in which a screening examination or more extensive clinical studies were made, and (2) dogs in which gross heart lesions were

seen at necropsy, but in which no cardiac examination was made prior to death. The latter group includes animals referred to the Department of Pathology of the University of Pennsylvania School of Veterinary Medicine for necropsy, and dogs dead on arrival at the small animal clinic.

Of the animals necropsied, 51 had been examined in varying detail through electrocardiography, radiography, clinical laboratory analyses, or other means. The postmortem and clinical diagnoses in this group are compared (table 5).

A 5-month-old dog with hepatomegaly, ascites, and electrocardiographic and radiologic evidence of right ventricular hypertrophy, had a systolic murmur in the pulmonic area. Angiocardiograms and cardiac catheterization indicated pulmonic stenosis. The diagnosis was confirmed at necropsy.

A 3-year-old Cocker Spaniel with polycythemia was thought to have a venous-arterial shunt on the basis of blood-oxygen saturation studies. Electrocardiograms and roentgenograms indicated right ventricular enlargement, but no murmurs could be detected despite repeated auscultation. The animal died before angiocardiograms could be made. At necropsy, a patent ductus arteriosus with a lumen as large as that of the aorta was found. The right ventricle was greatly hypertrophied. The flow of blood through this shunt was from right to left. Evidently, sufficient turbulence to produce a murmur was not present during the period this dog was under observation.

Chronic valvular disease was diagnosed

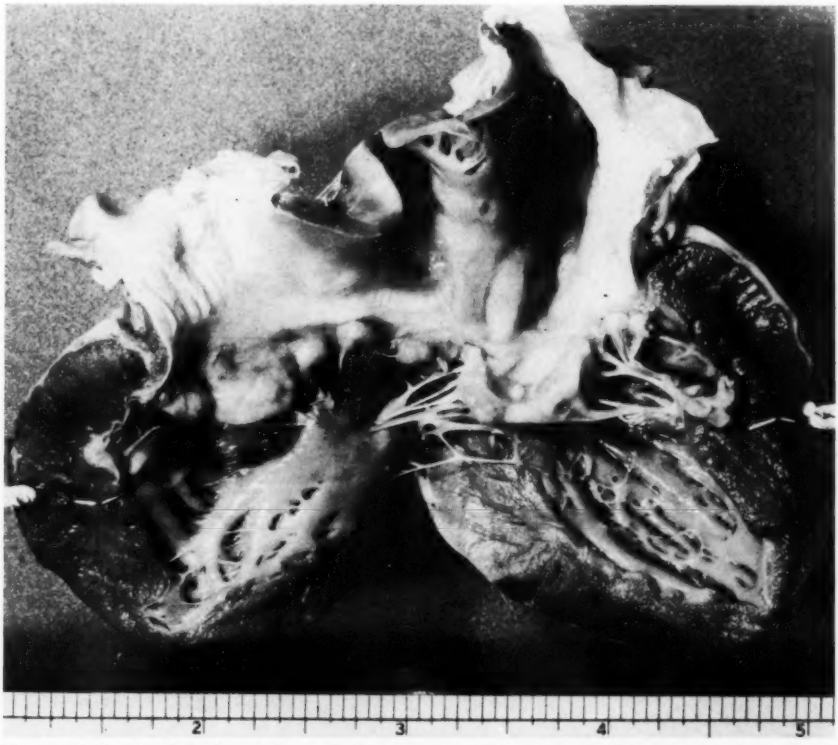


Fig. 3—Left side of the heart of a male dog, 11 years old, with marked thickening of the mitral valve cusps. During life there was a systolic murmur and a thrill present at the mitral area. Note the curled edges of the cusps and the opaque, wrinkled areas on the left atrial endocardium. The latter probably resulted from regurgitant streams (''jet'' phenomena) through the insufficient mitral valve. Microscopically, there was fibrosis of the valvular and mural endocardium at these sites (see fig. 4).

clinically in 10 dogs which had significant murmurs in the mitral or tricuspid areas, but no clinically detectable myocardial disease. On postmortem examination, 1 of these had valvular fibrosis alone. Seven had, in addition, varying numbers of small areas of myocardial degeneration or necrosis which accompanied thickening of small intramyocardial coronary arteries. A 2-year-old dog had congenital pulmonic stenosis as well as chronic valvular disease. In 1 dog, a low grade systolic murmur was heard at the mitral area, but no valvular or myocardial lesions were observed in the heart on postmortem examination.

Of 14 dogs which had clinical evidence only of myocardial disease (arrhythmias, conduction disturbances, RS-T segment deviations), 10 had myocardial degeneration and necrosis with coronary arterial thickening and minor degrees of fibrosis of the atrioventricular valves. Metastatic tumors were observed in 2, acute myocarditis and epicarditis in 1, and focal, purulent myocarditis in 1.

Clinical signs of chronic valvular disease, together with myocardial disease, were observed in 11 dogs. Both fibrosis of the atrioventricular valves and myocardial degeneration and necrosis with coronary arterial thickening were observed postmortem in 10. In one of these dogs, a patent foramen ovale, not suspected clinically, was seen at necropsy. The remaining dog had acute valvular endocarditis as well as myocardial lesions resembling the others in this group.

Signs of pericardial effusion were observed clinically in 1 dog which had acute myocarditis and epicarditis of unknown cause. A massive pleural effusion occurred in an 8-year-old German Shepherd Dog which had no other clinically detectable evidence of cardiac disease. At necropsy, a large aortic body tumor was found surrounding the anterior vena cava, pulmonary artery and aorta.

Enlargement of the right side of the heart due to disordered pulmonary circulation was determined clinically by means of roentgenograms, electrocardiograms, and palpation of the thorax in 2 dogs which had massive heartworm infestations.

A QRS axis considered abnormal was observed in the electrocardiogram of 1 dog which had no gross heart lesions when examined postmortem. This variation has since been shown to occur in normal dogs with certain chest conformations.

Nine dogs in which the screening examination revealed no evidence of cardiovascular disease had gross heart lesions when necropsied. Of these, 8 had minor degrees of fibrosis of the atrioventricular valves with myocardial and coronary arterial disease. One dog had myocardial lesions and arterial thickening but no significant valve changes.

TABLE 5—Comparison of Clinical and Postmortem Diagnoses in 51 Animals Necropsied

Clinical diagnosis	No. of animals	Postmortem diagnosis	No. of animals
Congenital pulmonic stenosis	1	Congenital pulmonic stenosis	1
Venous-arterial shunt	1	Patent ductus arteriosus	1
Chronic valvular disease	10	Chronic valvular disease	1
		Chronic valvular and myocardial disease	7
		Congenital pulmonic stenosis and chronic valvular disease	1
		No heart disease*	1
Myocardial disease	14	Chronic valvular and myocardial disease	10
		Metastatic tumors involving the heart	2
		Acute myocarditis and epicarditis	1
		Focal purulent myocarditis	1
Chronic valvular and myocardial disease	11	Chronic valvular and myocardial disease	9
		Patent foramen ovale and chronic valvular and myocardial disease	1
		Acute valvular endocarditis and chronic myocardial disease	1
Pericardial effusion	1	Acute myocarditis, epicarditis	1
Pleural effusion	1	Aortic body tumor	1
Cor pulmonale	2	*Dirofilaria immitis* infestation	2
QRS axis abnormality	1	No heart disease	1
No heart disease	9	Chronic valvular and myocardial disease	8
		Chronic myocardial disease	1
	51		51

* Not included in the 97 dogs listed in table 1, final distribution (category II).

The clinical diagnosis of chronic valvular disease was correct in 19 of 21 dogs necropsied. Only 1 animal in which a clinical diagnosis of chronic valvular disease was made had no valve lesions on postmortem examination. One had acute valvular endocarditis. Since all grossly visible valvular lesions were considered in the postmortem diagnosis, mild deformities of the valves, not advanced enough to produce insufficiency, account for the failure to detect valve changes clinically in some animals. The diagnosis of valvular lesions was based on the detection and location of murmurs on auscultation of the heart and, in some cases, on the palpation of thrills in the precordial region. In dogs over 4 years of age which had no signs of anemia or systemic infection, or evidence of congenital lesions, grade III or louder systolic murmurs in the mitral or tricuspid valve areas were considered diagnostic of chronic valvular disease.

Myocardial disease was detected clinically in 25 of 42 dogs which had myocardial lesions at necropsy, including acute and chronic lesions and metastatic tumors involving the heart. Most clinical diagnoses of myocardial disease were made on the basis of electrocardiographic abnormalities such as atrial fibrillation, ventricular extrasystoles, left bundle branch block, and RS-T segment deviations. Although only half of the dogs which had myocardial disease at necropsy had clinical evidence of the lesions, in each case in which a clinical diagnosis of myocardial disease was made, significant gross or microscopic lesions were observed on postmortem examination.

The group of dogs in which pathologic studies were made, but which had no cardiac examination before death, is of interest chiefly from the standpoint of the lesions observed, since clinicopathologic correlations are not possible in most cases. The necropsy material in this group was gathered from among all necropsies performed during the period of this study. The tis-

Fig. 4—A thickened mitral valve cusp from the heart of a male mongrel, 10 years old. The free edge of the cusp consists of proliferated fibroblasts and collagen fibers. There were numerous foci of myocardial necrosis throughout this heart. H & E stain; x 7.

sues of all dogs in which gross cardiovascular lesions were observed, were examined histologically.

Of 179 dogs necropsied consecutively, including a portion of those studied clinically as described above, and those which had gross cardiovascular lesions but had not been studied clinically, tissues from 69 were submitted for histopathologic study. The results of these pathologic examinations are summarized (table 6).

Thickening of the cusps of the atrioventricular valves was a common finding (fig. 3, 4). Examples of this lesion were noted in 39 animals. The mitral valve was involved in 36 of these. This lesion is nodular, smooth, firm, and usually located at the free margin of the cusp involved. In the more advanced examples, the cusps bulge convexly on the atrial surface. This serves to curl under the free margin of the cusp and to effectively shorten the chordae tendinae by bending under those attached at the margin. In many instances, it appears that the deformity, with the accompanying malformation and stiffening of the cusps, would have allowed regurgitation through the valve ring during life. The chordae tendinae are not thickened or altered in any other way. Microscopically, these cusps are thickened by fibrous proliferation with edema and mucinous degeneration (fig. 4).

TABLE 6—Summary of Principal Postmortem Findings in 69 Canine Hearts

Structures and lesions	No. of hearts
Coronary arteries:	
Thickened with narrowed lumen	54
Large thrombi	2
Emboli	1
Degeneration	6
Acute inflammation	4
Pericardium:	
Hydropericardium	3
Hemopericardium	2
Subacute inflammation	1
Myocardium:	
Acute inflammation	4
Abscess	1
Hemorrhage	13
Hypertrophy	9
Degeneration	27
Necrosis	17
Metastatic tumor	5
Endocardium:	
Acute inflammation	1
Subacute inflammation	2
Left AV valve:	
Fibrosis	20
Subacute inflammation	2
Cyst	1
Right AV valve:	
Fibrosis	3
Subacute inflammation	2
Cyst	4
Left and right AV valves:	
Fibrosis	16
Pulmonic valve:	
Acute inflammation	2
Cyst	1
Stenosis	2
Aortic valve	0
TOTAL FOR ALL VALVES	
Fibrosis	39
Subacute inflammation	4
Acute inflammation	2
Cyst	6
Stenosis	2

In contrast to the frequency of the fibrotic or chronic valvular lesions, acute inflammatory changes were encountered in only 2 cases; subacute changes, in 4 cases. In addition, subacute or acute mural endocarditis, not related to valvular inflammation, was observed in 3 hearts and acute myocarditis or abscess formation in 4. In 1 of the latter 4 hearts, acute inflammation of the pulmonic valve was also present.

Cardiac hypertrophy was diagnosed in 9 cases, 8 of which also had fibrotic thickening of the atrioventricular valves.

Heart weight:body weight ratios have

$$\left(\frac{\text{heart weight}}{\text{body weight}} \times 1{,}000\right)$$

been computed for 131 animals. Most of these cases (109) were from the group of 1,000 dogs screened, while 22 were necropsied later. The results are tabulated (table 7).

The 109 cases from the present material are represented in groups two and three (table 7) and are included in group one. Group four represents those cases, included in group three, which were diagnosed clinically. The mean relative heart weight was highest in the cases diagnosed clinically. In group two, the mean values for males and females were 8.3 and 8.2, respectively. Thus, a sex difference is not apparent.

Cardiac dilatation was diagnosed in 15 dogs, 5 of which also revealed foci of myocardial necrosis. Grossly, the diagnosis of cardiac dilatation was based on the finding of enlarged cavernous ventricular chambers, flattened papillary muscles, and thin ventricular walls. Microscopically, in dilatation the myocardial fibers are thin and certain groups are wrinkled or curled as though their attachment has ruptured. Cardiac dilatation was most frequently observed in the right ventricle and in many instances was thought to be a terminal episode.

Small foci of myocardial necrosis in various stages of resorption and scar formation were found in 17 canine hearts (fig. 5–8). These were areas of ischemic necrosis. The gross appearance of the lesions varied. Some were not visible or went undetected during gross examination. Depending on their age and degree of healing, other necrotic foci were reddish brown or gray and opaque. They varied in texture from soft and friable to firm and even gritty in some instances. Microscopically, these lesions are characterized by myocardial necrosis, infiltration by monocytes, fibrosis, and calcification of necrotic myocardium (fig. 8). Congestion and hemorrhage are often seen in and adjacent to the necrotic areas. There is some variation in size and etiology. Thrombosis or embolism by blood clots or tumor cells are some of the more apparent causes. Arteriosclerotic narrowing of arterial lumens is probably responsible for some others. The variation in size is undoubtedly related to the magnitude of the vessel occluded. The necrotic foci are not large, but frequently multiple and scattered throughout the myocardium. In 14 cases, the lesions were located in the myo-

Fig. 5—Surface of a longitudinal section through the posterior papillary muscle of the left ventricle of a 12-year-old dog. Notice the dark gray, irregular areas in some instances blending with the surrounding tissue, in others ending more abruptly. Microscopically, there was myocardial necrosis, hemorrhage, and monocytic infiltration. This was secondary to a tumor embolus occluding a branch of the coronary artery. x 3.

cardium of the left ventricular wall and anterior papillary muscle. The papillary muscle was not involved in 4 cases and 4 others were limited to the papillary muscle. Both left and right ventricle were involved in only 1 case and the change was limited to the right ventricle in 2 cases. This is an insufficient number of cases from which to draw any conclusions concerning sites particularly prone to this process.

Upon microscopic examination, 15 of these hearts also evidenced arterial thickening with narrowing of the lumen. The vessels changed were always small intramyocardial branches of the coronary arteries. Of the 17 hearts, 13 also revealed fibrous thickening of the atrioventricular valves.

Degenerative changes in the myocardial fibers less severe than actual necrosis were observed in 27 cases. The myocardium in these cases is pale, gray, and parboiled in appearance. Microscopically, the cells are swollen, the cytoplasm is granular, and the striations are not clear (cloudy swelling). Nuclear changes are not prominent in these cells.

The most commonly encountered lesion was arterial thickening, limited to small intramyocardial arteries. This microscopic

Fig. 6—Surface of a longitudinal section through the posterior papillary muscle of the left ventricle of a 7-year-old dog. The clinical diagnosis was pyometra and the animal died postoperatively. Notice the irregular, light gray areas, some of which have a lighter peripheral zone. This is a later stage of necrosis and organization than shown in figure 5. Microscopically, there is myocardial necrosis, congestion, monocytic infiltration, and arteriolarsclerosis. x 3.

lesion was seen in 54 of the hearts. In this series, the large surface arteries were free of lesions. Arterial thickening as a result of arteriosclerosis takes two forms; intimal and medial thickening. Degenerative changes are sometimes superimposed. The medial type of arteriosclerosis is characterized by markedly thickened walls with sharply reduced lumen size. This was seen in small arteries and arterioles, most commonly in the anterior papillary muscle of the left ventricle. The appearance of the media varies from a thick layer of fibers of smooth muscle and connective tissue elements which have numerous nuclei and appear hyperplastic to an almost anuclear hyaline material replacing and thickening the media (fig. 9). In some of the latter, there are free erythrocytes and mononuclear cells containing hemosiderin around the periphery and in the media of these arteries; the internal elastic membrane is largely replaced or fragmented in this type. The second type of arterial thickening re-

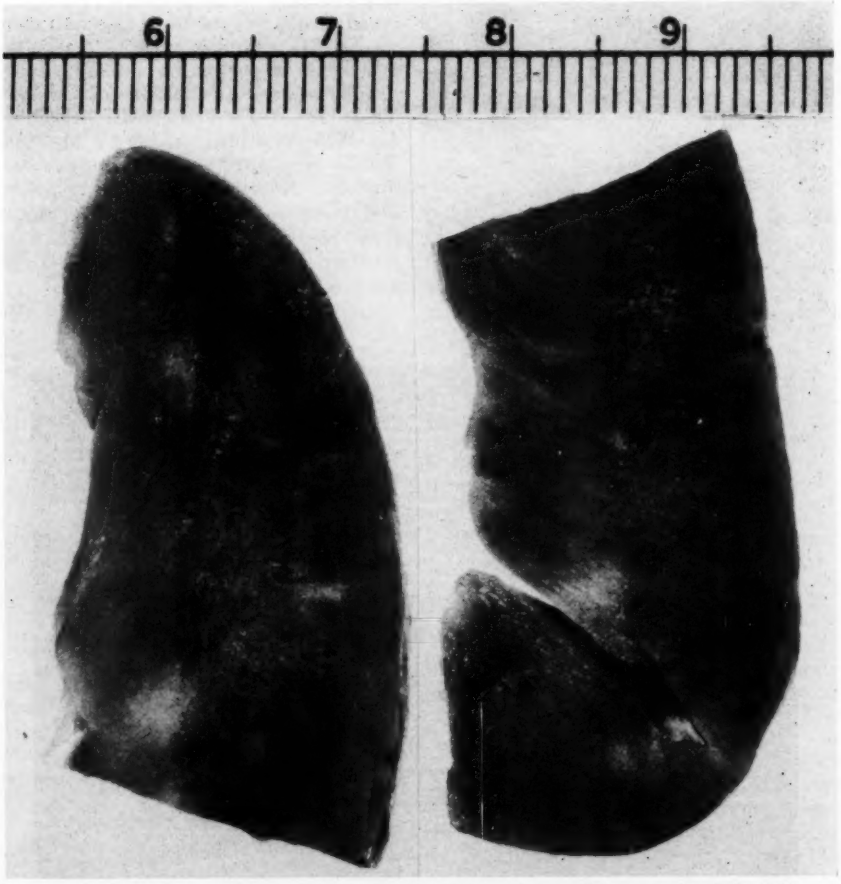


Fig. 7—Surface of longitudinal section through the posterior papillary muscle of the left ventricle of a 16-year-old dog. The clinical diagnosis was uremia. Notice the irregular, light gray areas which blend gradually with the surrounding myocardium. This is a later stage of myocardial necrosis and fibrosis than shown in figure 6. Microscopically, in these areas there was replacement of myocardium with fat and fibrous connective tissue, interspersed with monocytic leukocytes. A few necrotic myocardial fibers remained and there was capillary proliferation. Numerous small arteries and arterioles were thickened by medial hypertrophy, and in the left descending branch of the coronary artery there was an organized thrombus. x 3.

TABLE 7—Heart Weight/Body Weight Ratios x 1,000

Group	Number	Range	Mean	Standard deviation
1. Total hearts	131	5.2 to 17.9	9.0	2.06
2. No heart disease	57	5.2 to 13.6	8.3	1.61
3. Heart disease (all cases)	52	5.3 to 17.9	9.6	2.83
4. Heart disease (diagnosed clinically)	12	7.1 to 17.9	10.9	3.72

sults from intimal thickening. This occurs around the inside of the entire lumen and the internal elastic membrane is not as severely altered. The thickening in some instances is outside the internal elastic membrane as well as adjacent to the lumen. Of the 54 hearts in which arterial changes were found, the arteries of 19 were thickened as a result of intimal or medial changes. A thrombus occluded a small coronary artery in one heart and multiple emboli of thrombus origin were found in another.

Arterial cushions were frequently seen in small coronary arteries and arterioles. On cross section, these structures are usually wide at the base and taper to a rounded blunt tip, sometimes occluding much of the vessel. These cushions are eosinophilic, amorphous, covered by a layer of endothelium, and contain varying numbers of nuclei (fig. 10, 11).

The nuclei are round or oval on cross section, or oblong and oval on longitudinal section. They are either darkly basophilic or pale centrally, with no visible nucleoli. Elastic stains have demonstrated that the elastic membrane below these structures is fragmented and, in some examples, forms a network within the cushion. Some of the cases with arterioles thickened by medial hypertrophy reveal hyaline changes in these eccentrically placed bulges as well as in the media. In these instances, the elastic tissue is absent or condensed into an irregular-shaped mass near the base of the cushion. These structures are consistently negative for Weigert's fibrin stain. With Masson's Trichrome stain, they vary in their staining quality. If relatively heavily nucleated, and there is no hyaline change in the arterial media—the cushion stains like smooth muscle. If there is hyaline change in the arterial media, there is usually hyaline change with loss of nuclei in the cushion

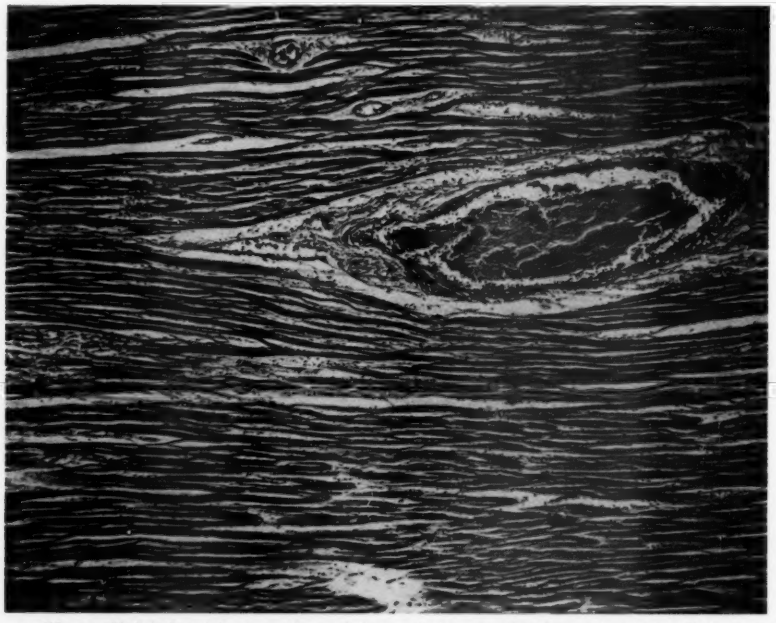


Fig. 8—Myocardial infarct in a section of the anterior papillary muscle of the left ventricle of the heart of a 10-year-old mongrel. Notice the embolus occluding the artery, necrotic myocardial fibers, and infiltrated monocytic leukocytes. H & E stain; x 151.

and it stains like hyalin, collagen, and connective tissue elements.

These structures are apparently smooth muscle, fibrous, and elastic tissue cushions arising from the arterial wall. The opinion expressed by Dahme [8] and Bucher [6] is that they are normal structures, containing longitudinally aligned smooth muscle. It is apparent from Dahme's [8] studies, that these cushions may show degenerative and sclerotic change when the arteries are diseased.

Such cushions were found in the small arteries in 35 hearts. The distribution in the left ventricle was in papillary muscles alone in 8 cases, only in areas other than papillary muscles in six hearts, and in 21 hearts they were in vessels in papillary muscles as well as other portions of the myocardium. They were present in the right ventricular myocardium in three hearts.

Sclerotic changes were seen in the aortas of 6 animals. Grossly, the lesions were pale yellow, raised, roughened, and firm; microscopically, they were seen to be the result of intimal thickening. They were most frequent in the abdominal aorta. All of these dogs also had thickening of the media in small branches of the coronary arteries as described. There were no lesions resembling atherosclerosis. Medial thickening of the arteries in other organs was also observed, but not as advanced as in the coronary arteries. Sclerosis of the pulmonary artery was seen in 6 cases. This lesion was visible by gross examination of the intima of the pulmonary trunk and the origin of the large branches. Microscopic examination of the smaller branches is often unrewarding owing to congestion or other lesions altering the tissue morphology. Of these dogs, 4 had fibrotic mitral valves and the fifth had tumor metastases in the lung. The sixth was unexplained.

Metastatic tumors were observed in the myocardium of five hearts. One aortic body tumor was found. Blood cysts or telangiectasis of the valve were seen in 6 dogs. Adult *D. immitis* were found in six hearts and there were 2 dogs with congenital pulmonic stenosis.

Types of Heart Disease Found

Among the 1,000 dogs examined, a total of 97 cases of heart disease were diagnosed (table 1). These diagnoses may be grouped in the following three categories: (1) undiagnosed, 3; (2) congenital heart disease, 4; and (3) acquired heart disease, 90. The 3 dogs with undiagnosed cases exhibited unmistakable signs of heart disease, but the exact lesion could not be identified with confidence. Three of the dogs with congenital heart disease were necropsied (table 5). One of them also had chronic valvular disease.

Fig. 9—Arteriosclerotic thickening and hyaline degeneration of a small coronary artery in the left ventricular myocardium of a 14-year-old male Pointer. Notice the markedly narrow lumen. Myocardial and valvular fibrosis were also present in this heart. H & E stain; x 184.

Of the 90 dogs with acquired heart disease, 46 were necropsied. There were 35 with chronic valvular disease and myocardial disease, 3 with acute myocarditis, 2 with metastatic tumors involving the myocardium, 2 with heartworm infestations, and 1 each with chronic valvular disease, chronic myocardial disease, acute valvular endocarditis together with myocardial disease, and aortic body tumor (table 5). The

remaining 44 cases were diagnosed clinically as follows: chronic valvular disease, 27; chronic valvular disease and myocardial disease, 11; cor pulmonale, 4; and myocardial disease, 2.

The most common types of acquired heart disease found were fibrosis of the valves (usually involving the mitral and tricuspid valves) and myocardial lesions. The latter included degeneration, inflammatory changes, and necrosis. These lesions were distributed throughout the myocardium in most instances. The areas of necrosis, some of which were in various stages of repair, were often associated with the vascular changes described under "Postmortem Examination."

Fig. 10—An arteriole in the left ventricular myocardium of a 9-year-old Cocker Spaniel. Notice the irregularly distributed nuclei and the amorphous area near the tip of the subendothelial cushion. This dog also had foci of myocardial necrosis, bronchopneumonia, and subacute nephritis. H & E stain; x 172.

In the necropsied dogs, chronic valvular disease and noninflammatory myocardial disease occurred together in 35 cases while valvular disease occurred alone in 1 and myocardial disease alone in 2 (table 5). This indicates that the myocardium is usually involved when chronic valvular disease is present in dogs necropsied. However, clinical evidence of myocardial disease was only detected in 13 dogs while a total of 38 cases of chronic valvular disease was diagnosed (table 4). This disparity between clinical and postmortem diagnoses is undoubtedly related, at least in part, to the difficulty of diagnosing myocardial lesions antemortem. Whether the chronic valvular, myocardial, and vascular lesions are etiologically related can only be conjectured at present.

The term *cor pulmonale* was used for those cases of right ventricular enlargement resulting from disordered pulmonary circulation.[19] Clinically, the diagnosis is based on the finding of electrocardiographic (fig. 12) and roentgenographic evidence of right ventricular enlargement and the presence of abnormally large pulmonary artery branches in thoracic roentgenograms (fig. 13, 14). Two dogs with signs of cor pulmonale were necropsied and heavy infestations of *D. immitis* found in the right side of the heart and pulmonary arteries.

Of the 97 dogs with heart disease, 6 had or developed unmistakable signs of congestive heart falure. In four instances, the clinical picture was dominated by signs of "right" heart failure; e.g., ascites, limb edema, liver enlargement. Two of these 4 dogs showed clinical signs of mitral and tricuspid incompetence (1 died and the diagnosis was confirmed on necropsy). The third dog had congenital pulmonic stenosis (fig. 15) and acquired valvular fibrosis with insufficiency of both atrioventricular valves, confirmed on necropsy. The fourth animal, which is still living, had clinical evidence of pulmonary hypertension with gross enlargement of the pulmonary arteries, probably the result of heartworm infestation.

The other 2 dogs with congestive failure showed clinical evidence of predominant "left" heart weakness (e.g., lung congestion, cough, dyspnea). Both dogs had clinical evidence of mitral valve incompetence

TABLE 8—Prevalence Rates of Heart Disease in Dogs of Various Age Groups

Type of disease	Age groups			
	0 to 2	3 to 6	7 to 10	11 and older
Chronic valvular disease	3.8	30.2	54.0	130.0
Myocardial disease	1.9	8.7	12.0	13.0
Chronic valvular with myocardial disease	0.0	30.2	126.0	220.0
Congenital heart disease	7.8	.....	.....	.....
Other	1.9	12.9	30.0	52.0
All types	15.4	82.0	222.0	415.0

(fig. 16, 17) and, on necropsy, fibrosis of the left atrioventricular valve was found.

The myocardiums of dogs which died of congestive heart failure showed areas of degeneration and necrosis and sclerosis and lesions of the coronary arteries.

AUSCULTATORY AND ELECTROCARDIOGRAPHIC ABNORMALITIES AND POSTMORTEM FINDINGS

Cardiac murmurs, various electrocardiographic changes, and roentgenographic abnormalities were detected in dogs subsequently necropsied, giving opportunity to evaluate the validity of diagnostic conclusions.

Murmurs.—Of the 97 dogs ultimately classified as having heart disease, 61 had systolic murmurs. No diastolic murmurs were detected in this group of 1,000 dogs (the 1 dog with patent ductus arteriosus did not have a murmur). Based on the auscultatory area in which the murmurs were most intense, the following clinical diagnoses were made: mitral insufficiency, 44 (fig. 16); mitral and tricuspid insufficiency, 10; tricuspid insufficiency, 5; pulmonic stenosis, 2. Of these 61 dogs, 21 were necropsied. In 19, valvular lesions corresponding to the clinical diagnosis were found. In several of these, other valves were also diseased. In 1 dog, no valvular lesions were present and in a dog with pulmonic stenosis and chronic atrioventricular valvular disease, a diagnosis of mitral and tricuspid insufficiency had been made but the pulmonic lesion had not been identified.

Electrocardiographic Changes and Conduction Disturbances (1) *Arrhythmias.*—When multilead electrocardiograms were taken, a number of abnormal arrhythmias and conduction disturbances were found which had not been detected during the screening examination or which developed later during the clinical course of the case. A total of 25 abnormal arrhythmias or conduction disturbances (fig. 18–21) were identified among the 1,000 dogs, distributed as follows: ventricular extrasystole, 6; incomplete atrioventricular block with dropped beats, 5; atrial fibrillation, 4; incomplete atrioventricular block, 3; paroxysmal ventricular tachycardia, 3; left bundle branch block, 2; right bundle branch block, 1; and paroxysmal atrial tachycardia, 1.

Ventricular Extrasystoles (fig. 18,B).—Four of the animals necropsied had frequent ventricular extrasystoles which were observed consistently in electrocardio-

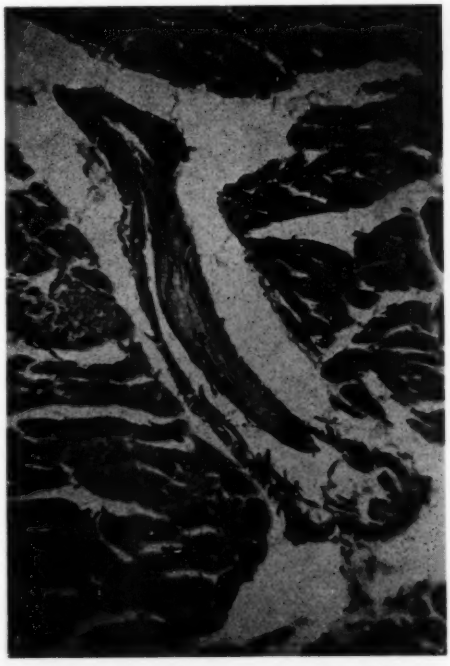
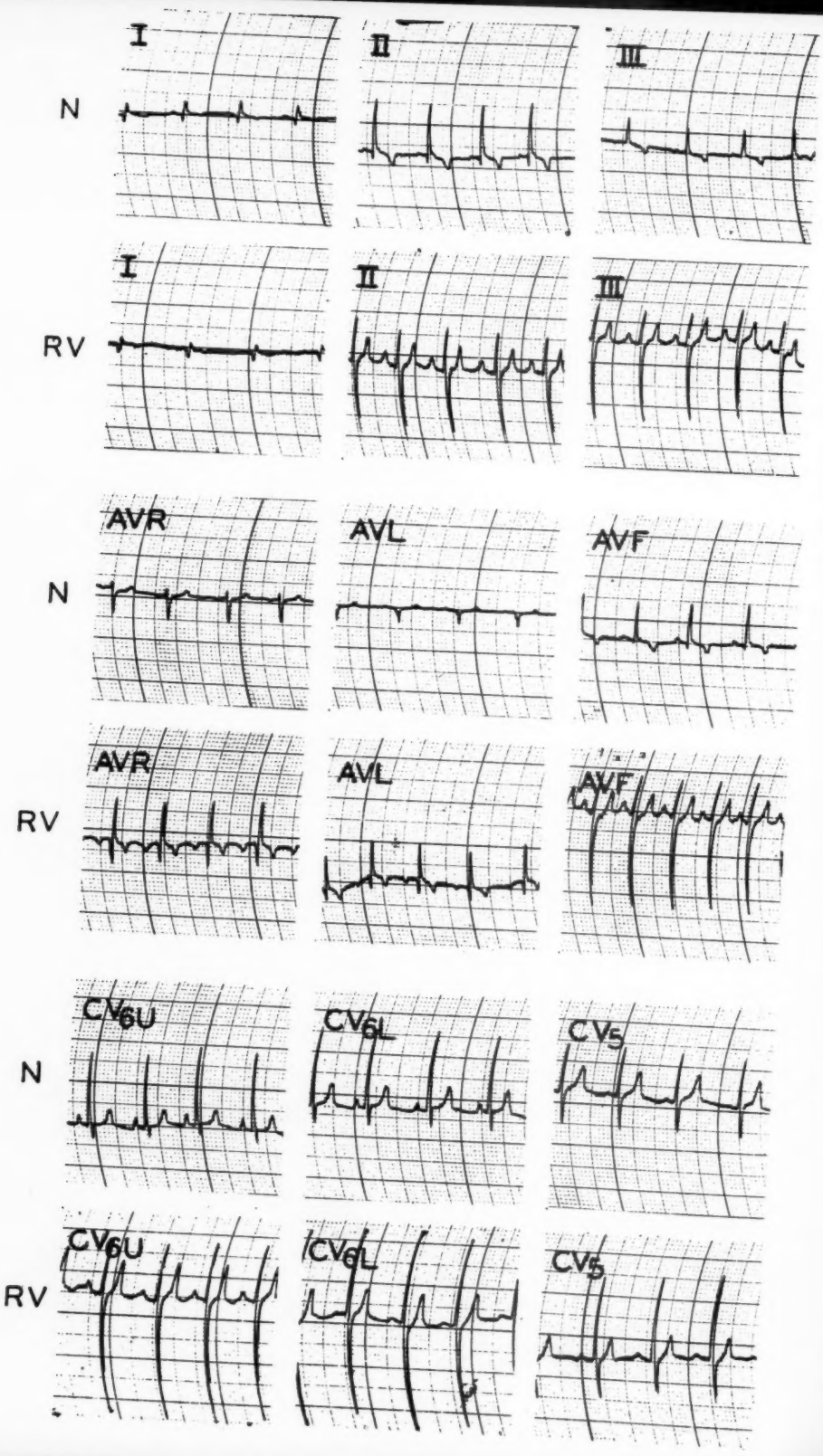


Fig. 11—A longitudinally sectioned arteriole and cushion in the left ventricular myocardium of a 7-year-old male Boxer. Notice the longitudinal streaks in the cushion, several oblong nuclei, and a layer of endothelium. There is a microfilarid in the vessel lumen. This dog also had foci of myocardial necrosis. H & E stain; x 120.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grams. Two of these exhibited both multifocal ventricular extrasystoles and bouts of paroxysmal ventricular tachycardia. One showed paroxysmal ventricular tachycardia and 1 had multifocal ventricular extrasystoles without paroxysmal tachycardia. Histopathologic studies showed coronary sclerosis, recent myocardial degeneration, and small healed infarcts in all 4 animals. Although a number of animals having similar myocardial lesions did not exhibit ventricular extrasystoles, it seems reasonable to attribute this arrhythmia to the myocardial disease. Ventricular extrasystoles are rare in dogs and available evidence [19] indicates that myocardial lesions are usually present when this arrhythmia is encountered under clinical conditions in dogs which have received no drugs or toxins known to cause ventricular premature beats. The 2 dogs having the most recent myocardial lesions exhibited bouts of paroxysmal ventricular tachycardia (fig. 18,D).

Atrial Fibrillation (fig. 18,A).—This arrhythmia occurred in 4 dogs which were subsequently necropsied. Three were old dogs (8, 13, and 14 yr.) which had advanced chronic atrioventricular valvular disease and myocardial disease characterized by cardiac dilatation, myocardial necrosis, and fibrosis associated with arterial thickening. All 3 had mitral insufficiency, left atrial dilatation, pulmonary congestion, and chronic passive congestion of the liver. The fourth dog had congenital valvular pulmonic stenosis as well as chronic valvular disease involving both the mitral and tricuspid valves. Myocardial degeneration was present but no arterial thickening was noted. There was hypertrophy and dilatation of the right ventricle, dilatation of the right atrium, chronic passive congestion of the liver, and ascites. Following treatment with strophanthin in this animal, atrial fibrillation converted to paroxysmal atrial tachycardia with variable incomplete atrioventricular block (fig. 21).

In these cases, atrial fibrillation occurred late in the course of advanced cardiac disease with congestive heart failure. Marked dilatation of one or both atria was present in all 4 dogs.

Bundle Branch Block.—Three animals with electrocardiograms interpreted as bundle branch block were found. The 2 dogs showing left bundle branch block (fig. 20) were necropsied, while the animal with right bundle branch block (fig. 19) had no evidence of cardiovascular disease and is in good health.

Electrocardiograms in all 3 dogs resembled recordings from dogs in which the bundle branches were cut experimentally.[21,37-40] QRS intervals are prolonged to within the range of tracings of experimental complete bundle branch block (0.06–0.08 sec.).[21] In both cases resembling left bundle branch block, Q waves in lead I and initial R waves in right precordial leads suggest normal initial left to right septal depolarization (fig. 20). These findings are not inconsistent with experimental left bundle branch block in the dog, however. According to Lepeschkin,[28] these deflections may be due to early forces in the right lateral wall of the right ventricle. Another possibility is that the block in the left bundle is distal enough that some left to right septal activation occurs. In man, if initial QRS forces are markedly rightward in direction prior to the development of left bundle branch block, the Q waves in lead I, and R waves in V_1 and V_2 may not disappear, but merely be shortened in duration and smaller in amplitude.[22]

No attempt has been made to classify the block as complete or incomplete in the dogs observed in this series. Bryant [5] states, "Complete bundle branch block can only be recognized with absolute certainty under experimental conditions by severance of the contralateral bundle branch to produce complete atrioventricular block. There are few clinical situations in which absolute

Legend for Figure 12 on Opposite Page

Fig. 12—Comparison of a normal canine electrocardiogram, and that of a dog with right ventricular enlargement whose roentgenogram appears in figure 13. The electrocardiogram from the normal dog is labeled N in each series; that of the dog with right ventricular enlargement is labeled RV. The clinical diagnosis in the abnormal dog was cor pulmonale, presumably secondary to heartworm infestation and associated pulmonary vascular disease. In the electrocardiograms labeled RV, note the large S waves in leads II, III, aVF, CV_{6U}, CV_{6L}, and the large R waves in leads aVR and aVL. Contrast the QRS complexes in the normal (N) and abnormal (RV) series.

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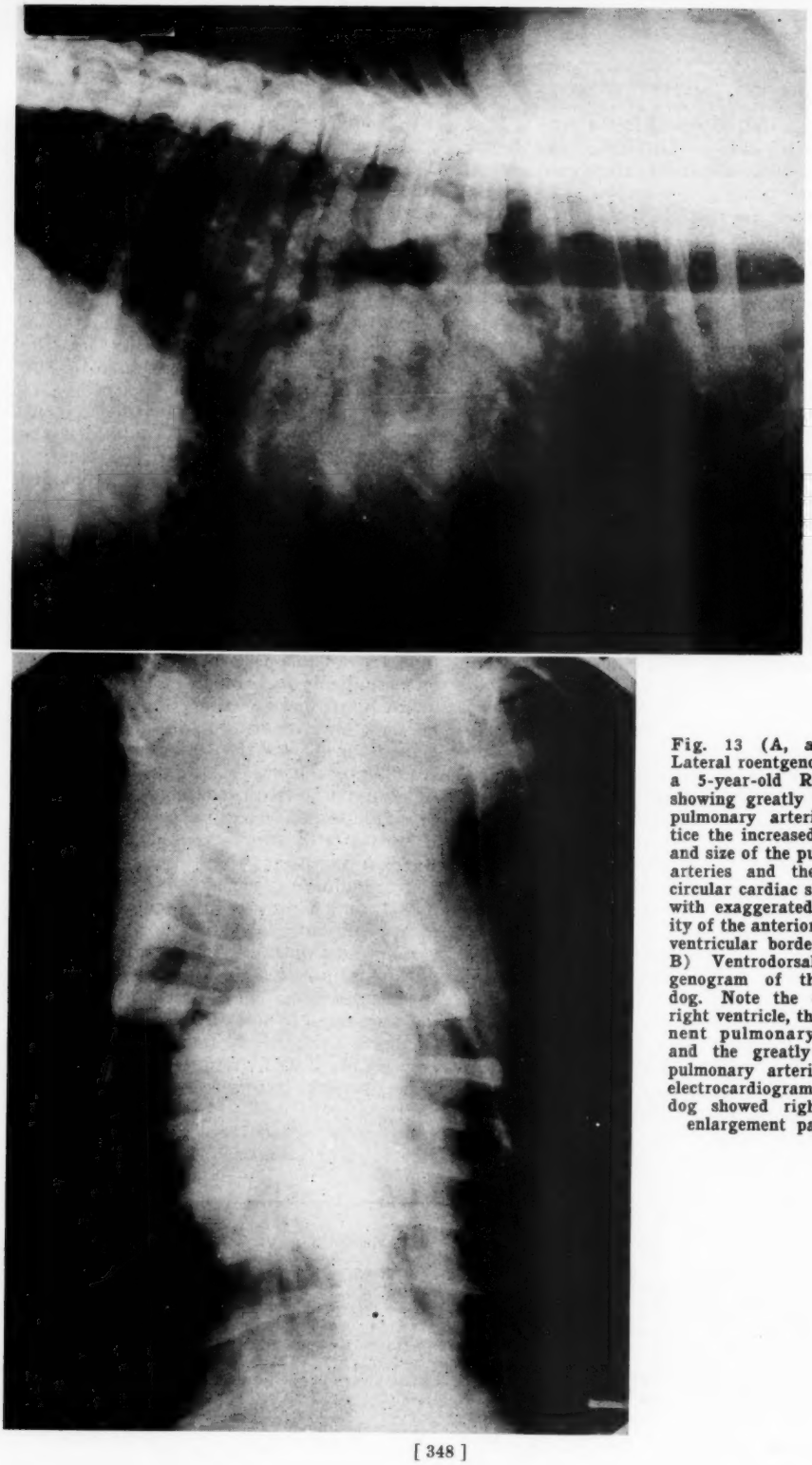


Fig. 13 (A, above)—Lateral roentgenogram of a 5-year-old Rottweiler showing greatly enlarged pulmonary arteries. Notice the increased density and size of the pulmonary arteries and the nearly circular cardiac silhouette with exaggerated convexity of the anterior (right) ventricular border.
B) Ventrodorsal roentgenogram of the same dog. Note the enlarged right ventricle, the prominent pulmonary conus, and the greatly dilated pulmonary arteries. The electrocardiogram of this dog showed right heart enlargement pattern.

evidence of the completeness of the block can be recognized." Authorities differ in opinion as to the mechanism, gradation, and the significance of various degrees of incomplete bundle branch block and satisfactory explanation has not been made for the wide range of QRS durations in "complete" bundle branch block in man.

Some experimental evidence [3] suggests that parietal focal blocks, in which lesions are located in the peripheral conducting tissue and myocardium of the ventricular walls, can simulate closely, right or left bundle branch block of the complete or incomplete type. Electrocardiographic differentiation in these cases might be made only by demonstrating an altered sequence of initial septal depolarization, as occurs in left bundle branch block.

Electrocardiographically, the 2 cases resembling left bundle branch block cannot be distinguished from lesions in the subdivision of the bundle or parietal block, on the basis of the limited electrocardiographic studies made. On microscopic examination, both hearts showed myocardial degeneration and fibrosis, arterial thickening, and valvular fibrosis. The clinical significance of the electrocardiogram in these cases (*i.e.*, myocardial disease) remains virtually the same, regardless of the location of the block.

2) *Deviation of the S-T Segment.*—Three animals having RS-T segment deviations of 0.2 mv. or greater in several leads were necropsied. Early myocardial degeneration and arterial thickening were present in all three hearts. Each had chronic valvular

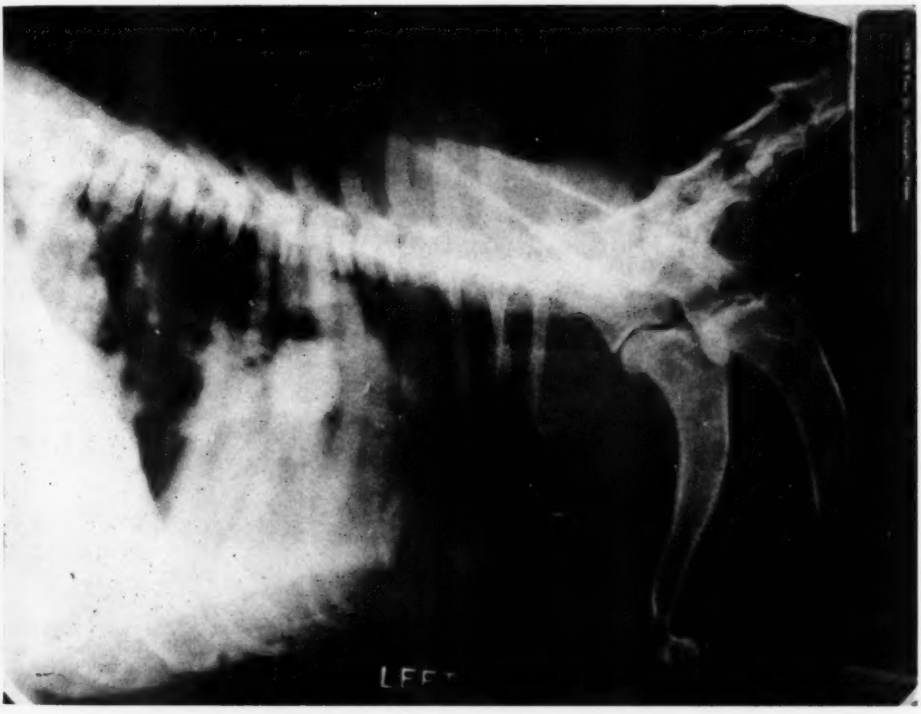
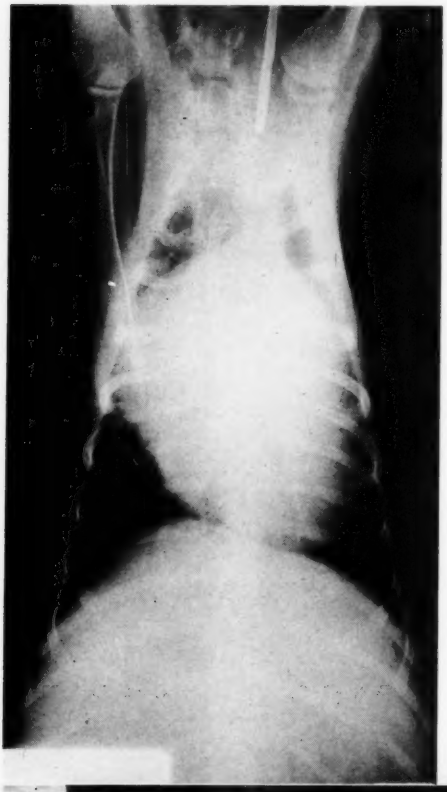


Fig. 14—Lateral roentgenogram of an 8-year-old Cocker Spaniel found at necropsy to have masses of heartworms occupying the right ventricle and pulmonary arteries. Many branches of the pulmonary arteries were occluded by thrombi formed around degenerating fragments of the parasites. Notice the large, radiopaque object at the base of the heart. This greatly dilated right pulmonary artery, viewed on end, has the appearance of a foreign body at first inspection. The anterior (right ventricular) border of the heart is abnormally full and rounded. The scattered areas of consolidation in the lung represent areas in which smaller pulmonary arteries were completely thrombosed. This dog had an electrocardiographic right heart enlargement pattern.

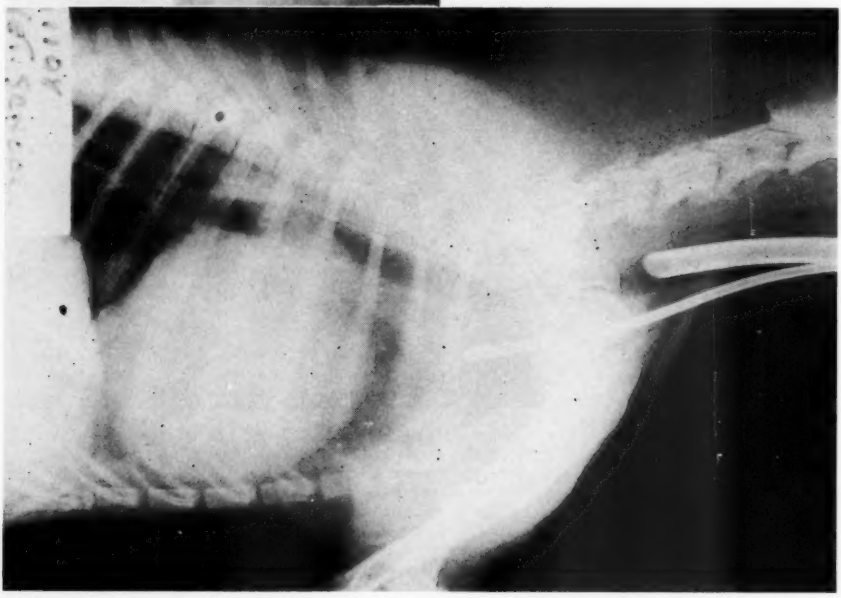


disease involving one or both atrioventricular valves.

3) Right Heart Enlargement Pattern (fig. 12, 21).—This electrocardiographic pattern was observed in 4 dogs which later were necropsied. Two of the dogs had congenital stenosis of the pulmonic valve, 1 had a large patent ductus arteriosus with shunting of blood from the pulmonary artery to the aorta, and in the fourth, masses of heartworms were present in the right ventricle and pulmonary arteries. All four hearts had definite right ventricular hypertrophy, evidenced by marked thickening of the right ventricular free wall.

The electrocardiograms from these dogs were characterized by tardy QRS forces directed toward the right, resulting in marked deviation of the mean electric axis of the QRS complex to the right and by

Fig. 15—Ventrodorsal (left) and lateral (below) roentgenograms of a dog with pulmonic stenosis. Notice the prominence of the right heart border in the ventrodorsal view and the full, rounded appearance of the anterior (right ventricular) border in the lateral view. The conspicuous bulging of the lower half of the posterior heart border in the lateral view represents the left ventricular wall, which has been displaced upward and posteriorly by the enlarged right ventricle. Endotracheal and intravenous catheters can be seen in the cervical region.



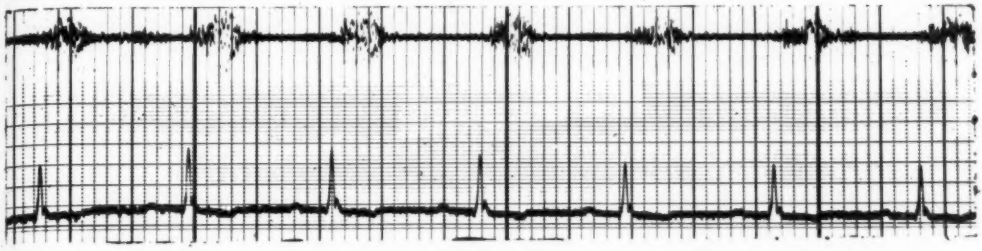


Fig. 16—Simultaneous phonocardiogram (upper tracing) and electrocardiogram from a 6-year-old Boston Terrier with mitral insufficiency. This grade V systolic murmur was audible at some distance from the animal. The recording was made with the microphone over the mitral valve area.

the presence of large S waves in precordial leads over the left ventricle. The mean electric axis of the QRS complex among the 4 dogs varied from −150 degrees clockwise to −90 degrees. Lannek [27] found the statistical mean in 230 normal dogs to be +79 degrees ± 24.6 degrees (range −27 degrees clockwise to −150 degrees; *i.e.*, a total spread of 237 degrees) and Lombard *et al.*[29] reported that in 90 per cent of 50 anesthetized dogs, the mean QRS axes ranged between 41 and 120 degrees.

4) Incomplete Atrioventricular Block.— Of the 8 dogs with spontaneous atrioventricular block, 5 had dropped beats (fig. 18 C) while 3 merely had prolonged P-R intervals. None of these animals were necropsied. Subsequent studies suggest

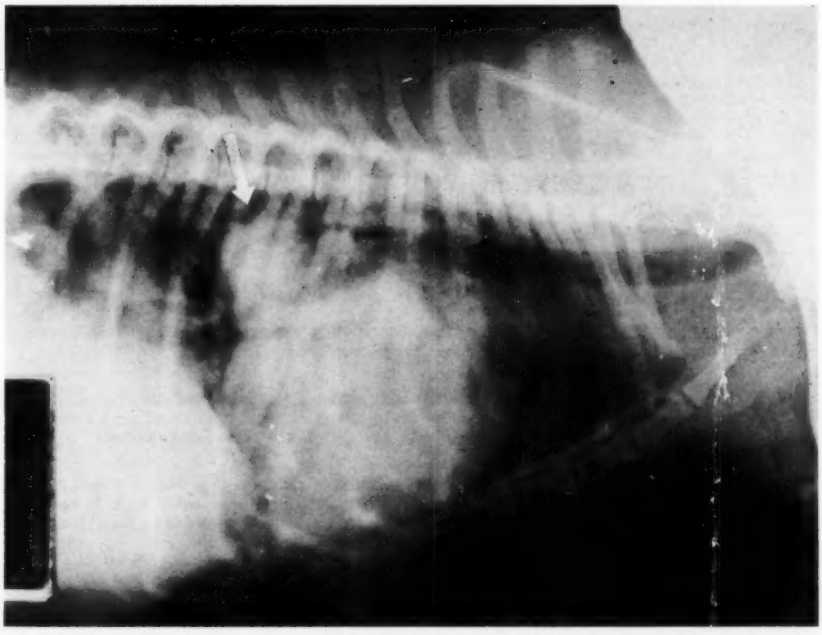


Fig. 17—Lateral roentgenogram of a 10-year-old Cocker Spaniel with long-standing, decompensated, mitral insufficiency. Notice the greatly enlarged cardiac silhouette, the prominence of the dilated left atrium (arrow), and the severely congested lungs. The animal was euthanatized several months after this roentgenogram was taken. Marked fibrosis of the mitral valve, hypertrophy and dilatation of the left ventricle, dilatation of the left atrium, and pulmonary congestion and edema were found at necropsy.

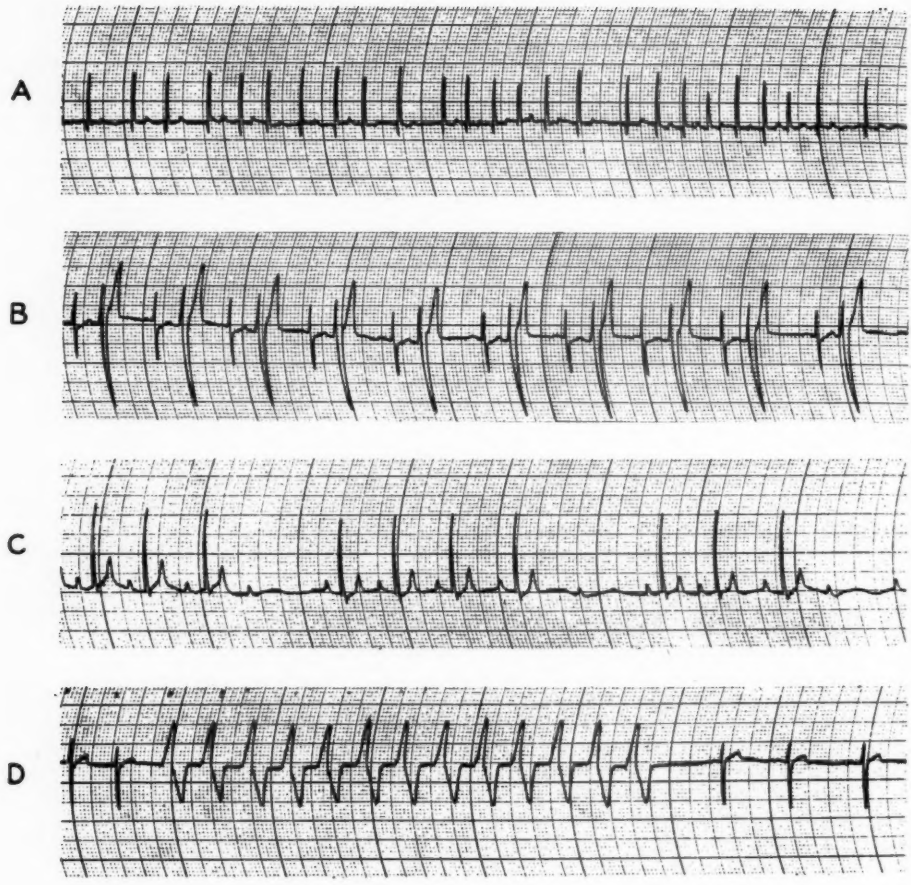


Fig. 18—These arrhythmias and conduction disturbances occurred spontaneously abong the first 1,000 dogs screened.

A) Lead II—atrial fibrillation in a dog with mitral and tricuspid valve insufficiency. Notice the absence of P waves, the presence of small undulations of the base line (fibrillation waves), and the irregularity and variation in form of the ventricular complexes.

B) Lead CV_5—these coupled ventricular extrasystoles occurred in a dog with systemic bacterial infection. Notice that the extrasystoles (large, bizarre QRS complexes with high T-waves) are coupled to the previous normal beat by a constant interval (fixed coupling). In this case, the extrasystoles disappeared after treatment with antibiotics.

C) Lead II—incomplete atrioventricular block with drooped beats in a dog with no other clinically detectable cardiac abnormality. Although incomplete heart block with dropped beats can be considered an electrocardiographic abnormality in the dog on a statistical basis, it may or may not be associated with clinically significant cardiac disease.

D) Lead CV_5—paroxysmal ventricular tachycardia in a dog with severe anemia associated with a hemolytic bacterial infection. The electrocardiogram returned to normal after clinical recovery.

that this disturbance may occur in animals with no other signs of cardiac abnormality. Animals which developed incomplete atrioventricular block under the influence of digitalis glycosides were not considered in the "spontaneous" category.

5) *Low Amplitude Electrocardiographic Potentials.*—Two dogs with low-amplitude electrocardiograms were necropsied. One had a heart base tumor with pericardial and pleural effusions secondary to venous obstruction. The second dog had acute myocarditis and epicarditis with an inflammatory pericardial effusion. Low-amplitude potentials occurred in 2 other necropsied dogs which did not have heart disease. One had extensive, granulomatous pleural and pericardial lesions with pleural effusion, and in the second widespread pulmonary metastatic tumors with extensive consolidation of the lungs were found.

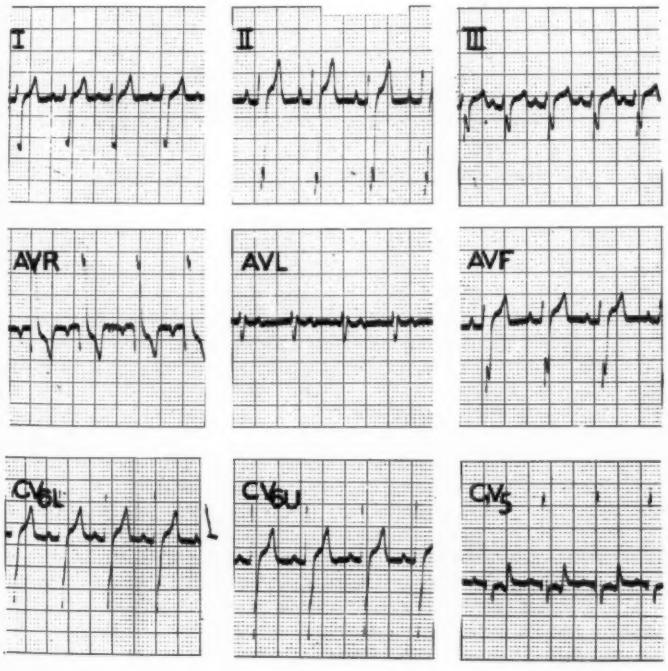
In the first 3 dogs, the low amplitude electrocardiograms apparently resulted from the interposition of a fluid medium between the heart and the recording electrodes. In the last case, abnormally dense lungs, filled with metastatic lesions, may have had the same effect.

AGE SPECIFIC PREVALENCE OF HEART DISEASE

Table 8 gives the age-specific prevalence rates of heart disease in dogs based on the 1,000 dogs included in this study. The expected rates of the various types of heart disease were computed for four age groups from the 97 dogs finally placed in category II, heart disease.

As can be seen from the table, the prevalence of all types of acquired heart disease is lowest in the 0 to 2 age group, and increases with age, there being a sharp rise in the 7- to 10-year group. In the animals over 11 years of age, 415 dogs in every 1,000 would be expected to have some form of cardiac disease.

Fig. 19—Right bundle branch block occurred in a 2-year-old Airedale with no other evidence of cardiovascular disease. Notice that the QRS interval is prolonged (0.08 sec.). The major (widest) QRS deflection is negative in leads I, II, III, aVL, aVF, CV_{6L}, and CV_{6U}, and positive in aVR and CV_5, indicating delayed right ventricular depolarization. An rsR′ complex is present in lead CV_5. This pattern is characteristic of that found in experimental bundle branch block in the dog (section of the right bundle branch).[21, 37, 39–41] The prominent initial R deflection in the leads having widened, notched S waves is attributed by Wilson and associates to an early activation of certain portions of the left ventricle.[40]



In man, right bundle branch block patterns are usually characterized by negative major QRS deflections in leads I and II, and positive major QRS deflections in lead III. Patterns resembling those experimentally produced in the dog may occur in persons having a vertical heart position.[23]

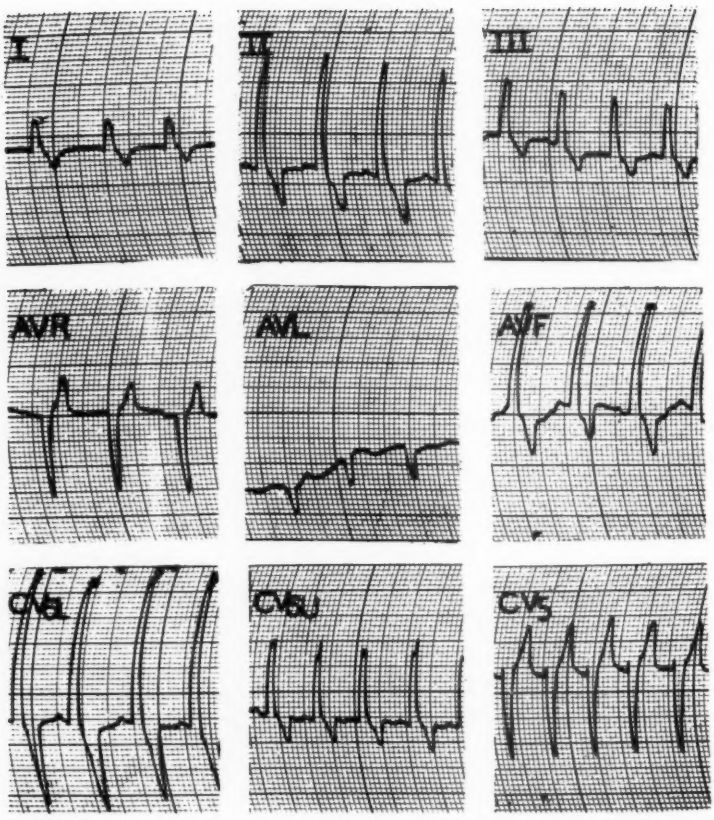


Fig. 20—This conduction disturbance, resembling left bundle branch block, occurred in an 11-year-old English Bulldog. In addition to aortic and mitral valve fibrosis, numerous foci of myocardial necrosis throughout the ventricles were observed at necropsy. Histopathologic examination of the heart revealed medial thickening, hyalinosis, and thrombosis of small coronary arteries with ischemic necrosis of small areas of myocardium. The delayed conduction presumably resulted from the effect of extensive myocardial disease on the left bundle branch or its main subdivisions. Notice that the major portion of the QRS complex is prolonged (exceeds 0.07 sec.) and is upright in all three standard limb leads, aVF, and leads over the left precordium, while it is inverted in a aVR, aVL, and CV_6. This pattern is characteristic of experimental left bundle branch block in dogs (section of the left bundle branch).[21, 37–39] Notice that a small Q wave is present in lead I and a small R wave in CV_6. Although initial QRS forces in left bundle branch block are classically in a right-to-left rather than the normal left-to-right direction, such a small Q_1 is often present in experimental left bundle branch block in dogs.[39]

In left bundle branch block in man, the major deflection of the QRS complex is usually upright in lead I and inverted in lead III (discordant), although in individuals with a vertical heart position, the QRS may be upright in leads I, II, and III as in the dog.[28]

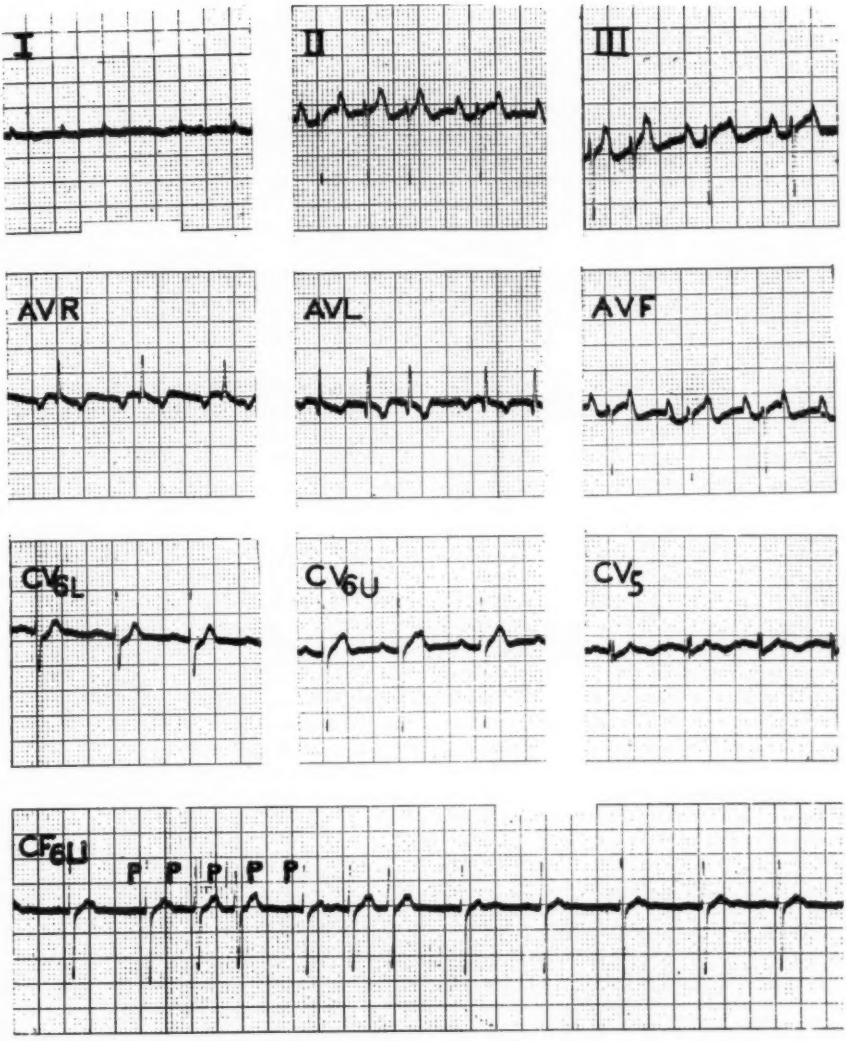


Fig. 21—This tracing shows right heart enlargement pattern and paroxysmal atrial tachycardia with varying degrees of AV block. These abnormalities occurred in a 2-year-old German Shepherd Dog cross found at necropsy to have congenital stenosis of the pulmonic valve, chronic atrioventricular valve disease, and marked right ventricular hypertrophy. The arrhythmia occurred after treatment with strophanthin and conversion from atrial fibrillation. Notice that in parts of the record only every other P wave is followed by a ventricular complex, the blocked P wave being superimposed on the preceding T wave in these instances. Occasionally a 1:1 response occurs and the ventricular rate doubles for a few beats (see fig. 12 for description of right heart enlargement pattern).

Congenital heart disease is found only in the 0 to 2 age group in this series, the expected rate in the group being 7.8 dogs per 1,000. One 13-year-old dog in which a patent foramen ovale was found, incidentally, was not included in the computation.

Chronic valvular and myocardial disease constitute the principal types of cardiac disease occurring after 2 years of age, and increase in prevalence with age. The sharp rise in the 7 to 10 age group is due to the increased prevalence of chronic valvular and myocardial disease at this age. In the group of dogs over 11 years of age, the prevalence further increases. A group of 1,000 dogs over 11, presented to our clinic, would be expected to contain 130 individuals with chronic valvular disease and 220 would be expected to have both chronic valvular and myocardial disease.

The group "other" (table 8) included heartworm disease *(D. immitis)*, heart base tumors, metastatic tumors involving the heart, and undiagnosed cases. The prevalence rate in this category also increases with age.

Necropsy findings (table 5) indicate that valvular and myocardial lesions are present in a higher number of dogs than is clinically evident. The prevalence rates (table 8) are based on clinical as well as postmortem findings and clinically "silent" cardiac lesions such as minor degrees of chronic valvular and myocardial disease were not detected in dogs not necropsied. A study based entirely on postmortem evidence would undoubtedly reveal a higher prevalence rate in all age groups than that reported here. For the same reason, the almost invariable co-existence of chronic valvular and myocardial disease in the same hearts, which was evident in the dogs necropsied, is not reflected in table 8.

DISCUSSION

In this survey, the screening methods employed revealed abnormalities in 15 to 20 per cent of the animals examined and the presence of cardiovascular disease was confirmed by further clinical or postmortem study or both in about half of these. The correlation between clinical diagnoses and postmortem findings indicates the clinical examination methods are effective and do not result in many false positive diagnoses, but regularly miss a few individuals with heart disease.

The three most useful diagnostic procedures employed in recognizing heart disease in dogs were auscultation, electrocardiography, and roentgenography. Owing to the high prevalence of valvular disease, auscultatory abnormalities were the most frequent found. Thus, auscultation is the most useful method in detecting heart disease, since both murmurs and abnormal arrhythmias can be recognized. Electrocardiograms and roentgenograms were of about equal usefulness in establishing a diagnosis of heart disease. Ideally, complete electrocardiograms and lateral thoracic roentgenograms should be employed in screening dogs for heart disease, but cost and time limitation precluded this in the present study. Certainly in dogs suspected of having heart disease, thorough auscultation, multiple-lead electrocardiograms, and thoracic roentgenograms should be employed, together with other diagnostic procedures.

Clinically, the most commonly detected abnormality was a systolic murmur at the cardiac apex. This was usually associated with chronic disease of the mitral valve and consequent insufficiency. When the murmur was of grade III or greater intensity, the diagnosis of mitral incompetence was often supported by other findings (*i.e.*, roentgenographic evidence of cardiac enlargement) and there has been good correlation between clinical and postmortem diagnoses.

It is well known that certain dogs have such systolic murmurs for several years without developing other clinical signs of heart disease. Unfortunately, there are no data available on the length of time such murmurs have been present in dogs which do develop congestive heart failure. Experimentally induced mitral insufficiency in the dog has not proved a reliable means of producing chronic congestive heart failure [31] and experimental studies have shown that in the normal heart an extremely large regurgitant flow has relatively little influence on effective cardiac output, aortic pressure, and left atrial pressure.[4] It appears that two factors interact to account for the degree of cardiac disability which develops in a given case of mitral insufficiency: (1) the volume of regurgitant flow, and (2) the condition of the myocardium. Since the former will increase with elevation of the cardiac output, the amount of exercise to

which an animal is subjected will also play a role.

Our findings indicate that a combination of chronic atrioventricular valvular disease and myocardial disease is usually present in dogs developing clinical evidence of chronic congestive heart failure.

The chronic valvular lesions are characterized by fibroblastic proliferation and the 39 cases examined in this series showed no histologic evidence of inflammation. However, the prevailing view is that they represent the healed stage of an inflammatory process, probably of bacterial origin.[35] Somewhat in support of this explanation was the finding of six hearts with acute and subacute inflammatory valvular lesions out of a total of 45 with pathologic valves. Our observations neither refute nor confirm the hypothesis that the chronic valvular changes result from an acute bacterial process. Since the chronic change in these valves is essentially a proliferative process and owing to the absence in this series of progressive intermediate stages which would make it clear that the acute lesions lead to the chronic ones, the pathogenesis of the chronic valvular changes cannot be established.

The myocardial lesions often found associated with chronic valvular disease consist of small necrotic foci apparently caused by interference with the blood supply. In some cases a clot or embolus was seen occluding a nearby vessel. These lesions are considered true infarcts. Other foci resemble these lesions except that an occluded vessel cannot be found. It is possible that further sections would reveal an occluded vessel in at least some of these cases. In all instances, arterial thickening with narrowing of the lumen is seen. Perhaps the resultant relative reduction in blood supply is sufficient to account for the myocardial necrosis observed. Polymorphonuclear leukocytes in large numbers are usually found in myocardial infarcts during the early stages. However, in these small necrotic foci observed in the dog, polymorphonuclear leukocytes are rare or absent. The predominant cells found are mononuclear leukocytes and macrophages. For the foregoing reasons, some of these necrotic areas do not appear to be true infarcts.

Coronary artery disease was confined to small intramyocardial arteries. Lesions were observed in 54 of the 69 hearts examined histologically. True atherosclerosis was not seen in any of the hearts and the large superficial coronary arteries were free of pathologic changes. The small vessel disease was usually associated with degenerative or necrotic changes in the myocardium, suggesting that the vascular alterations resulted in myocardial damage owing to interference with the blood supply.

The endothelial cushions, which in some cases encroach extensively on the lumen of small coronary vessels, were fairly common in certain hearts. Their relationship to myocardial disease is not clear. Obviously these structures cause narrowing of the lumen of the vessels in which they are found and any pathologic change in a cushion or underlying arterial wall which would exaggerate this narrowing effect would reduce the flow of blood. It has been suggested that these cushions may have some normal regulatory function.[26] However, the nature of this possible function is not understood and thus the influence of disease is difficult to assess on the basis of present information.

The occurrence of bundle branch block is of interest because this conduction disturbance has only been reported once previously in the dog.[34] In this case, the electrocardiographic pattern was that of left bundle branch block. The dog had congestive heart failure and, on postmortem examination, myocardial degeneration was found. From the evidence thus far available, it appears that in the dog, as in man, left bundle branch block pattern is usually associated with myocardial disease while right bundle branch block pattern not infrequently occurs in individuals with no evidence of heart disease.

As has been emphasized elsewhere,[19] cor pulmonale occurs in dogs as a clinical entity characterized by typical electrocardiographic and roentgenologic signs. The electrocardiographic changes are the result of a shift of the mean electrical axis of QRS to the right (fig. 12). This may occur whenever the right ventricle is enlarged, irrespective of the cause. In the cases of cor pulmonale reported here, the cause was heartworm infestation. From studies made on dogs with adult *D. immitis* in the right heart and pulmonary arteries, it is apparent that pulmonary hypertension [33] associated with pulmonary arterial changes (atherosclerosis, endothelial thickening, endothelial cushions, medial hypertrophy)[25] occur and account for the right ventricular

enlargement and failure. In addition to the electrocardiographic changes, enlargement of the right ventricle and pulmonary arteries can be detected in thoracic roentgenograms of dogs with severe heartworm disease. Other causes of right ventricular enlargement producing similar electrocardiographic changes include pulmonic stenosis, tetralogy of Fallot, patent ductus arteriosus with pulmonary hypertension and right to left shunting of blood, or any other lesion resulting in selective hypertrophy of the right ventricle.

Although a few individuals with arterial hypertension, persisting after attempts to accustom the animal to the procedure of blood pressure measurement, have been encountered, none of these have been followed long enough to establish the diagnosis and none have died. A relationship between chronic renal disease, arterial hypertension, and arteriosclerosis is evident from the studies of others,[8,19] but at the present time our data do not contribute to the observations already made. One of the problems is the time required to obtain reliable resting blood pressure determinations in untrained animals. Because of this, blood pressure determinations have not been made on many of the animals examined in this series.

In determining cardiac hypertrophy, the use of heart weight/body weight ratio may be misleading, especially in individual cases, owing to the great variation in normal animals. Recently, Stünzi and coworkers[36] have pointed out that body length is a more suitable reference than body weight for this purpose and Marthaler,[30] working in Stünzi's laboratory, has reported, for the dog, data emphasizing the great breed variations in relative heart size and shape.

SUMMARY

Of 1,000 dogs screened by clinical methods for the presence of cardiovascular disease, 97 were found to have heart disease after confirming clinical and postmortem studies. An additional 80 dogs had signs suggesting cardiac abnormality which could not be confirmed at the time of study.

The clinical screening methods consisted of palpation of the cardiac region; auscultation of the heart; palpation of the femoral arteries; and a single lead, precordial electrocardiogram. Auscultation and electrocardiography were the two most effective screening procedures in detecting abnormalities, while palpation of the cardiac region served, in only a few cases, to reveal abnormalities which otherwise would have been missed. Palpation of the femoral arteries was negative in this series. For practical purposes, the three most useful diagnostic methods employed in recognizing heart disease were auscultation, electrocardiography, and roentgenography.

The most common types of heart disease found were: (1) chronic valvular fibrosis with deformity and consequent insufficiency; (2) myocardial disease including necrosis and degeneration with associated sclerosis of the deep coronary vessels; (3) right ventricular dilatation and hypertrophy associated with *Dirofilaria immitis* infestation; and (4) congenital heart disease.

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Calcium and Phosphorus Absorption and Its Relationship to Growth in the Dog

D. H. WILL, D.V.M., M.S., and R. H. UDALL, D.V.M., Ph.D.

Fort Collins, Colorado

NORMAL HEALTH can be maintained throughout growth and even for a lifetime on different dietary levels of calcium and phosphorus, yet differences within the range of normal may ultimately influence the degree of health and well-being of the individual. From the many studies made in the field of nutrition, it is evident that there is a difference between the merely adequate diet and the optimum diet.

This study was undertaken to ascertain the degree of association between a high potential ability for new bone formation, as in growth, and the per cent of net absorption of calcium and phosphorus at two dietary levels.

REVIEW OF LITERATURE

The metabolic influence of dietary calcium and phosphorus has received a great deal of attention since the early work on rickets by Mellanby.[13] Extensive studies[2, 17, 18, 21] have been made of the effects of different dietary levels of calcium and of phosphorus upon the growth rate of rats. The levels of calcium studied varied from 0.2 per cent to 0.8 per cent of the dry weight of the feed, with a calcium to phosphorus ratio of 1.5:1. From these studies, it was concluded that the optimal level of calcium in the diet reached a plateau between 0.65 and 0.8 per cent and the calcium to phosphorus ratio should be 1:1 to 1.5:1. Sherman[17] defined as "adequate" those diets which contained about 0.2 per cent calcium and which would support normal growth without signs of bone abnormalities. He defined as "optimum" those diets which contained sufficient additional calcium to give a maximum growth stimulus; the plateau for this effect of added calcium was between 0.65 per cent and 0.8 per cent. Carttar *et al.*[3] studied the effects of Sherman's "adequate" and "optimum" diets upon the formation and structure of bone in rats. They found that in rats given the optimum diets, the metaphysis was relatively longer and more dense, bone trabeculae calcified to a greater density with no osteoid borders, and the shaft was more dense. The morphologic bone differences between the rats on the two diets were most extreme at about 7 weeks of age and diminished with time. Early growth, as measured by body weight and linear body measurements, was greater on the optimum diet, but the differences between the two diets decreased as growth progressed.

The true mechanisms by which calcium and phosphorus are absorbed from the gastrointestinal tract are not clear. Greenberg[6] believed that the absorptive mechanism of these two ions was one of simple diffusion and therefore depended upon their concentrations in the intestine. Harrison and Harrison[8] have reported that the absorptive mechanism for calcium could be divided into two phases. They observed an initial rapid absorption from the proximal part of the small intestine and postulated that this was due to simple diffusion. The second and slower phase of calcium absorption was from the distal small intestine, where it was believed that the concentration of calcium would be low due to a higher pH value, and the process here depended upon an active transport mechanism. Considerable indirect evidence exists in support of the hypothesis that calcium and phosphorus absorption is proportional to their concentrations in the intestine.[5, 7, 11, 15, 16]

MATERIALS AND METHODS

This study was carried out with purebred Beagle dogs given adequate and optimum dietary levels of calcium and phosphorus as defined by Sherman.[17] Nine dogs from two groups of littermates, all males except 1, were started on the diets at about weaning time and continued for approximately nine months. The dogs were divided into two dietary groups consisting of 4 and 5 animals each.

The diets differed mainly in the amounts of calcium and phosphorus. Dogs were given a level of 0.3 or 0.8 per cent calcium, on a dry weight basis, with a ratio of calcium to phosphorus of 1:1. The composition of the two diets is presented (table 1). The diets were made up in 50-kilogram lots as needed. The eggs were mixed with the starch and cornmeal and cooked before adding the remaining ingredients.

The individual feed intake was recorded daily and measurements were made weekly on individual weights. A fecal sample was collected twice weekly from each dog for chemical analysis. Net absorption was measured with chromic oxide as a reference substance as described by Kane *et al.*[10]

From the College of Veterinary Medicine, Colorado State University, Fort Collins.

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A wet ashing procedure was used on the feed and fecal samples.[1]

The calcium content of both the feed and fecal matter was determined following precipitation as the oxalate by titrating with ceric ammonium nitrate in perchloric acid as described by Smith.[20] Fecal and dietary phosphorus were determined colorimetrically by phosphovanadmolybdate method of Simonson *et al.*[19] Chromium was determined colorimetrically, as described by Bolin *et al.*,[1] from a direct aliquot of the digestion mixture using a blank from the unknown solution to correct for the dichromate present. Net absorption was determined by the following formula:

$$\text{Per cent net absorption} = 100 - \frac{(\text{fecal ratio}^* \times 100)}{(\text{feed ratio}^*)}$$

Two male dogs, 1 from each group, died in the early part of the study from canine distemper and all information obtained from them was consequently deleted from the final results. Another male dog, although used throughout the entire study, was much retarded in growth. This was not due to insufficient feed intake. The condition was thought to be hereditary in origin. Subsequent necropsy revealed an intussusception of the lower small intestine with partial occlusion; as a consequence, the data obtained on this dog were also omitted from the final results.

RESULTS

The growth data (fig. 1), for the first 11-week and final 25-week periods were analyzed by use of Student's "t" test. There was no significant difference in the growth rate between dogs in the two groups. Dogs in both groups, by the end of the study, had reached a similar plateau of maturity.

Analysis of data on absorption disclosed that the average net absorption of calcium was 30.4 per cent for those dogs on the low dietary level and 42.2 per cent for those on the higher dietary level. For phosphorus, the average net absorption was 50.1 per cent and 68.8 per cent for the low and high dietary levels, respectively.

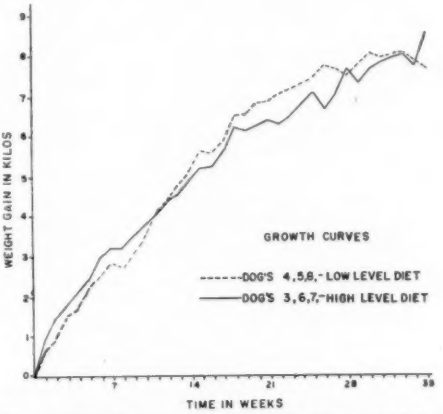

Fig. 1—Growth curves of dogs fed diets containing either 0.3 or 0.8 per cent calcium, with a calcium to phosphorus ratio of 1:1.

The coefficients of correlation of the net absorption of calcium and phsophorus, as well as the regressions of net calcium and phosphorus absorption, on growth for both levels of feeding were tabulated (table 2). Although both correlation coefficients were significant at the 1 per cent level, the coefficient for the low level was by far the larger.

DISCUSSION

The results indicate that under the conditions of this study the dietary level of calcium and phosphorus had no demonstrable effect upon growth of the dog. This finding does not coincide with that of Sherman and coworkers[2,17,18,21] or with that of Carttar *et al.*,[3] who showed an increase in the early growth rate of rats on diets considered as optimum in calcium and phosphorus over those on only adequate levels.

Why a liberal dietary supply of calcium and phosphorus might act as a stimulus to early growth, as in the foregoing studies

* Ratio = Ca/Cr of P/Cr.

TABLE 1—Composition of Diets and Mineral Mixture Given to Dogs

Ingredient	0.3% Ca and P (Gm./kg. of diet)		0.8% Ca and P (Gm./kg. of diet)
Eggs (shelled)	330.0		330.0
Corn (meal)	330.0		330.0
Casein	50.0		50.0
Starch	240.0		220.0
Yeast	10.0		10.0
Cod liver oil	20.0		20.0
Sodium chloride (iodized)	5.0		5.0
Chromic oxide	3.0		3.0
$CaHPO_4 \cdot 2H_2O$	1.55		25.0
$CaCO_3$	3.45		0.8
Mineral mixture	7.0		6.2
Potassium chloride		250.0	
Magnesium sulfate		150.0	
Ferric sulfate		50.0	
Potassium aluminum sulfate		0.2	
Copper sulfate		0.4	
Sodium fluoride		0.4	
Manganese sulfate		0.2	
Cobalt chloride		0.1	
Zinc chloride		0.1	

Composition of the mineral mixture is given in grams.

TABLE 2—Net Absorption of Calcium and Phosphorus in Dogs

Calculation	Low level diet (0.3% Ca–P)	High level diet (0.8% Ca–P)
Correlation coefficient "r" Ca:P	0.532*	0.170*
Regression of calcium absorption on weight (y is % net absorption, x is weight in kg.)	$y = 56.96 - 3.5x$	$y = 45.27 - 0.35x$
Regression of phosphorus absorption on weight (y is % net absorption, x is weight in kg.)	$y = 60.29 - 1.52x$	$y = 69.69 + .083x$
"t" value of slope of net calcium absorption on weight regression	6.25*	0.14
"t" value of slope of net phosphorus absorption on weight regression	3.80*	0.35
Average net absorption (%)		
Calcium	30.4	42.2
Phosphorus	50.1	68.6

* Significant at the 1.0 per cent level.

with rats, is not entirely clear. It suggests that growth is a characteristic limited by the minimal levels of dietary essentials and also that the requirement changes with age. Certainly, there is little doubt that for most species the early phase of growth is the period where the demands for calcium and phosphorus are the highest and that they decrease progressively as the individual approaches maturity.

It is conceivable that species variation may account for the difference recorded in the dog and rat. It is also possible that the two dietary levels of calcium and phosphorus studied do not represent adequate and optimum intakes for the dog. In addition, the limited number of experimental subjects used in the study was a probable influencing factor.

The average net absorption of calcium and phosphorus in dogs on the high-level diet was greater by 11.8 and 18.5 per cent, respectively. When based on absolute levels of intake, this actually represents an increase in net absorption of about 3.7 times as much calcium and 3.6 times as much phosphorus.

The results of this study suggest that the dietary levels of calcium and phosphorus do have an important influence on their absorption, probably by virtue of their altered concentrations in the intestine. This suggestion is in accord with both Greenberg [6] and Harrison and Harrison.[8]

Although it would appear that the passage of calcium and phosphorus through the intestinal wall is more than a process of simple diffusion, absorption by this means seems to play a major role.

The correlation coefficient r (0.532 for calcium and phosphorus absorption at the low-level diet) suggests a rather close relation between the mechanisms by which each was absorbed. It should not necessarily be interpreted as meaning that both calcium and phosphorus were absorbed by the same mechanism. In a review on calcium and phosphorus metabolism, Greenberg [6] stated that the absorption of these two ions was to a large degree independent of each other. The decrease in the value of r to 0.170 at the higher level of feeding suggests that, at this level, factors (such as absorption by passive diffusion) were increasingly important and that factors operating in common at the lower level were most apparent only at that level.

The regression equations indicate an inverse relationship between the change in absorption and the change in weight. At the low level of intake, this change is more apparent in the larger values of β or slope, than for the higher levels. At the low levels of intake, the slope of the regression equation is more dependable than at the higher level which is commensurate with the correlation coefficients. As the dietary calcium and phosphorus increased, the relationship between net absorption and body weight became less predictable.

The lack of a clear relationship of net absorption to weight gain at the higher dietary level indicates that the total net absorption remained more nearly constant and that probably storage of calcium and phosphorus was greater. This latter assumption appears reasonable when one considers that there is no apparent regulated excretion of calcium back into the intestine [14]; that the normal urinary excretion of calcium in the dog is small [4]; and that, although the normal renal loss of the phosphate ion appears to be considerable,[9] substantial amounts of this ion must necessarily be deposited with calcium in the bone. Indeed, recent studies in young dogs by Liu and McCay [12] showed that both calcium

and phosphorus retention values could be increased by elevating the dietary levels of these two ions.

SUMMARY

A study was made to determine the effects of two dietary levels (0.8 and 0.3%) of calcium and phosphorus at a ratio of 1:1 upon growth of the dog. The percentage of net absorption of calcium and phosphorus at the two dietary levels was also determined.

Neither the 0.8 per cent nor the 0.3 per cent dietary levels of calcium and phosphorus had an apparent advantage or disadvantage on the growth rate.

The average net absorption of calcium and phosphorus was greater at the high level of feeding. It was postulated that there was greater retention of calcium and phosphorus at the high level of feeding and hence greater bone deposition of these two ions.

Negative regression of both calcium and phosphorus net absorption on growth was more pronounced at the low level. This indicated that greater net absorption was maintained at the higher level as the animal matured, and the degree of saturation of the bones in these individuals should have been greater.

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Absorption of I^{131}-Labeled Fat in the Dog

SOL M. MICHAELSON, D.V.M.; M. Y. EL-TAMAMI, M.S., M.D.;
R. A. E. THOMSON, M.T.; JOE W. HOWLAND, M.D., Ph.D.

Rochester, New York

IN ORDER to determine the normal sequence of fat absorption and metabolism, various indicators or markers have been employed. Since ordinary quantitative chemical analytical methods of metabolic functions are tedious, the use of isotopes has been found advantageous. Fats labeled with stable isotopes H^2 and C^{13} or radioactive isotopes C^{14} and I^{131} have been utilized in studies of fat metabolism, since these elements are easily traced and can be measured with a high degree of accuracy.

The use of hydrogen or carbon isotopes is considered ideal because no foreign atoms are added to the fat molecule. Their assay, however, is technically more difficult than when I^{131} is used as a tracer. It has been fairly well established that, although iodized fat is unnatural, its digestion, absorption, and metabolism are not unlike that of natural fat. The iodine label appears to be stable and to remain fixed on the fat molecule through digestion, absorption, and transport of the fat to the storage depots. Fats labeled with I^{131} have frequently been used in clinical and experimental studies of normal and abnormal patterns of fat digestion and absorption, and their usefulness and reliability have been confirmed.

Hoffman[1] demonstrated the stability of the iodine label on the fatty acid through digestion, absorption, and transport to the storage depots.

Rutenberg *et al.*[2] administered I^{131}-labeled soybean oil to dogs. They found that, as the fat was metabolized, I^{131} accumulated in the blood as organic iodide. Stanley and Thannhauser,[3] using I^{131}-labeled olive oil in humans, determined that the rate of absorption was similar to that of natural dietary fats.

The following experiments were performed in order to characterize the pattern of fat absorption in the normal dog. This is part of a larger program undertaken to characterize fat absorption in dogs under various physiologic situations.

MATERIALS AND METHODS

In this study, a radioiodinated glycerol * preparation was used. This was administered in capsule form with a fixed amount of the neutral fat and an asayed total I^{131} activity per capsule. Capsules were found to be more convenient, easier to handle and administer, and better for technical reproducibility than the emulsion which is usually used. This method eliminated possible variations in the quantity and physical form of the meal.

Healthy mongrel dogs (4 females and 3 males), 1 to 5 years old, were used. Food was withheld from the dogs for about 18 hours before the test. Fifteen drops of Lugol's iodine was given on the morning of the test. This was done to block the accumulation of the inorganic radioiodine liberated from the metabolized radioiodinated fat into the thyroid gland. The capsule containing the test meal was introduced by hand into the pharynx of the dog to assure successful ingestion. The total amount of the bound I^{131} activity in a test meal was 50 μc. Using heparinized syringes, blood samples were collected from the jugular vein at hourly intervals for six hours after ingestion. During this period, the dog received water but no food. A blood sample collected before giving the test dose served as a background reference.

The radioactivity in the blood was estimated as counts per minute per milliliter of whole blood, using aliquots of the blood counted for a sufficient total number of counts to give a standard error percentage of less than 1 to 3 per cent. The total activity of the ingested test meal in counts per minute was calculated using a suitably diluted sodium radioiodine reference solution. All the blood samples collected during a test were saved and counted simultaneously with the background blood sample and the standard I^{131} sample at the end of the test to obviate the effect of the physical decay of the I^{131}. Counting was carried on throughout the study using a thallium-activated, sodium iodide crystal scintillation counter of the well-type

From the Department of Radiation Biology, University of Rochester School of Medicine and Dentistry, Rochester, N.Y. Dr. Michaelson is assistant professor, Mr. Thomson is research associate, and Dr. Howland is professor of radiation biology, University of Rochester Atomic Energy Project. Dr. Tamami, a WHO fellow at the University of Rochester Atomic Energy Project, is a radiologist, Institute of Research and Hospital for Tropical Diseases, Cairo, Egypt.

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* Trioleotope, produced by E. R. Squibb and Sons, New Brunswick, N.J.

with a counting efficiency of 40 per cent for a 1-ml. sample.

The most valid correction for variation in size of animals is considered to be the calculation of activity for the total blood volume of each animal.[4] Blood volume was calculated directly as a fixed percentage of the body weight, using the rounded figure of 100 ml. of blood per kilogram of body weight.[2] The determination of body weight under the fixed condition of a fasting state on the day of test tended to diminish variations in blood volume.

The activity estimated in the blood at hourly intervals for six hours after ingestion was calculated as a per cent of the total dose ingested per total blood volume. These values were plotted against time (in hours) on a linear scale. Where an assayed dose of I^{131}-labeled fat is fed to a fasting animal and the percentage of this dose recovered in the blood is estimated in a serial fashion in the fasting state, the curve obtained may be considered as a tolerance-like curve that can be used for comparing the results obtained.

Results

The greatest part of the activity determined in the blood of these dogs was built up very rapidly during the first two hours after ingestion (fig. 1).

One hour after ingestion, 6.7 ± 4.1 per cent * of the ingested I^{131} was determined to be active in the blood. These one-hour levels are indicative of gastric emptying.

At two hours after ingestion, activity in the blood was 13.6 ± 2.0 per cent. The values at this time, which is the end of the most rapidly rising phase of the curve, are indicative of the rate of fat absorption, digestion, and distribution to the blood.

The highest values reached in these 7 dogs varied from 13.1 to 21.2 per cent, with an average of 16.1 ± 2.5 per cent. The degree of variability among the first-hour values was greater, suggesting that the variation in normal dogs is more evident when the gastric emptying function occurs than when other functions, especially absorption, occur. The variation of the time of gastric emptying did not affect the net values as long as there was complete evacuation and a chance for the other processes to act.

After two hours, the rate of recovery slowed markedly through the third hour to merge into a variable plateau extending with a very slow rise or, following a peak value, a gradual fall until the end of the sixth hour. These curves indicated that the absorption of the ingested fat from the intestine occurred largely during the first two hours.

* Mean ± standard deviation.

Discussion

The term, fat absorption, as used in these studies on intact animals, includes the entire sequence of events for the given meal from the time of ingestion to the time the activity present in the blood was estimated. In this sense, the values obtained and the curves represent physiologic fat absorption. This includes (1) the time and rate of passage of the fat from the stomach to the duodenum, (2) the rate of its transit through the small intestine, (3) the efficiency of the digestive and absorptive functions, (4) the patency of the transporting channels to the blood, and (5) the rate of removal of the circulating fat from the blood to deposition in metabolic sites.

The curves obtained in this experiment have certain characteristics which can be used for a comparison of the results. It has been indicated by Turner [4] that the responses of normal dogs to an I^{131}-labeled triolein meal, although varying quantitatively within the normal range, were usually found to be reproducible responses when tested on separate occasions under similar conditions. The shape and maximum height of the curve in any single normal animal can thus be considered as a reliable index for evaluation of physiologic changes.

In general, the effect of the time and rate of gastric emptying will be reflected on the shape and the relative position of the rapidly rising phase of the curve and not on the net values of the heights reached. Where there is a sluggish rate of gastric emptying extending over the whole observation period,

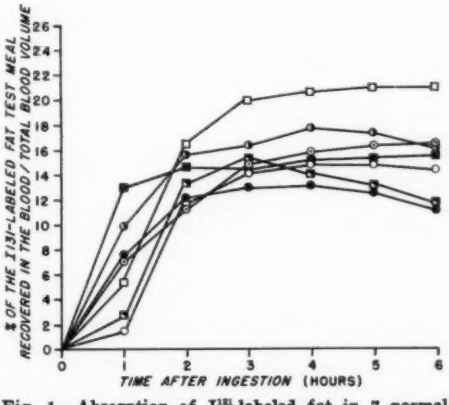

Fig. 1—Absorption of I^{131}-labeled fat in 7 normal dogs.

a markedly flattened curve with a slow gradual increase will be observed, even if digestion and absorption are normal.

With complete gastric evacuation, the amount of fat recovered at the end of the rapidly rising phase of the absorption curve will be mainly decided by the efficiency of digestion, the absorptive capacity of the mucosa, and the time the meal stays in contact with the absorbing region of the intestine. Absorption is considered as a continuous process wherein the end products of digestion are continuously removed from the intestinal lumen by the intestinal cells of the absorbing regions, and then enter the blood. Defective digestion or absorption, or effective rapid transit of the meal through the intestine, would cause varying degrees of lowering or flattening of the building-up curve, depending on the degree of this defect. The flattened curve would, however, have a limited maximum height followed by a gradual fall, unlike the curve produced by a slow but continuous gastric emptying.

After ingestion there is normally a continuous supply of fat into the blood, thus raising the activity values in the blood. In addition, there is a continuous removal of this fat from the blood to the deposition and utilization sites. Studies carried on by Turner [4] in normal dogs, using intravenously administered I^{131}-labeled triolein emulsion, revealed that the amount of lipid-bound I^{131}, estimated after being separated from the free inorganic iodine liberated from the utilized fat, falls rapidly after administration. This indicates that normally the circulating fat is rapidly removed from the blood. Where the total radioactivity of the blood is measured as a whole without the separation of the inorganic I^{131}, the resultant curve would not fall as rapidly.

As long as the rate of absorption of fat into the blood exceeds its rate of removal from the blood, the curve will rise. The peak value is reached where both rates are equal, after which the rate of removal of the circulating fat predominates. Accordingly, the maximum heights of the curve will depend on the relative effect of both processes.

In the present study, the variability of the values of activity determined at the end of the first hour could denote a variability in the gastric emptying time within that period. Except for 2 dogs that showed values of 1.4 and 2.7 per cent, the first-hour values were usually in excess of 5 per cent of the administered dose. This represents delayed gastric emptying. These 2 animals were the youngest of the group, both 1 year of age. Variability in gastric emptying due to the effect of age on the tone of the pylorie sphincter may be deduced from these results.

Turner [4] reported a difference between young and aged normal dogs in their response to I^{131}-labeled triolein administered orally or intravenously. The values of the plasma lipid activity determined after injection were markedly higher and maintained for a longer time in the aged dogs than in the young ones. This would indicate a slower rate of removal of fat from the blood. It is likely that the high fat-tolerance curves of the aged dogs was due to this decreased removal of fat rather than to increased absorption. This slower fat disappearance may be related to a defect in the rate of entry of fat into the tissues or presence of a metabolic deficiency, since no clinical or detectable histopathologic changes were reported which could account for this observed difference between both groups.

SUMMARY

Using an I^{131}-labeled neutral-fat test meal, the fat absorption pattern for normal dogs has been characterized and described.

There appears to be a difference in gastric emptying time between young and old dogs, with a delay noticed in the younger dogs.

Within two hours after ingestion of the fat meal, intestinal digestion, absorption, and distribution to the blood is practically complete.

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The Effects of Feeding Irradiated Beef to Dogs. I. Growth

ELWOOD F. REBER, Ph.D.; OM P. MALHOTRA, M.S.; JULIUS P. KREIER, V.M.D.; HORACE W. NORTON, Ph.D.; PAUL D. BEAMER, D.V.M., Ph.D.

Urbana, Illinois

TREATMENT of foods with ionizing radiation promises revolutionary advances in food preservation techniques. The irradiation levels used by most investigators have been 2.79 or 5.58 megarads. Irradiation with 5.58 megarads does not affect the apparent digestibility or biologic value of beef.[3] Poling *et al.*[5] fed raw ground beef irradiated with 1.9 megarads to three generations of rats. The beef was stored in a frozen state until it was included in a ration. The data from this experiment indicated that irradiation did not significantly impair the nutritional value or wholesomeness of beef.

The effects of feeding nonirradiated or irradiated beef to dogs during their growth period is the subject of this report.

MATERIALS AND METHODS

Ground beef, packed in No. 10 cans containing approximately 2.5 kg. of beef, was packed commercially as directed by the Quartermaster Food and Container Institute for the Armed Forces. The nonirradiated portion of beef (B0) was shipped from Chicago, in the frozen state, to Urbana where it was stored at −18 C. until used. The treated beef was irradiated by the Phillips Petroleum Co., Idaho Falls, Idaho. The No. 10 cans of beef were irradiated with either 2.79 megarads (B3) or 5.58 megarads (B6); were shipped without refrigeration; and, after arrival in Urbana, were stored at 22 to 27 C. for 36 weeks, after which they were held in a freezer at −18 C. until needed. The proximate analysis of the beef was: 40.5 per cent total solids, 17.1 per cent protein, 19.8 per cent fat, and 0.8 per cent ash.

When needed for ration preparation, the beef was thawed and autoclaved in open containers for 45 minutes at 20 lb. of pressure (127 C.) and was incorporated at a level of 35 per cent of total ration solids. The composition of the ration is shown (table 1). Mixed rations were stored frozen for one to 14 days and were fed within 24 hours after thawing. The dogs were fed twice daily. At each feeding they were given the amount of food they were able to consume within half an hour. Daily feed consumption records were maintained. Feed efficiency was calculated on the basis of feed composition at the time it was mixed. The proximate analysis of the ration as fed was 64.8 per cent total solids, 13.8 per cent fat, 5.0 per cent ash, 18.3 per cent protein, and 2.2 per cent crude fiber.

Vitamins A, D, and E were given orally in capsules once per week in doses of 5,000 I.U. of vitamin A, 500 I.U. of vitamin D, and 30 mg. of vitamin E per 4 lb. of body weight.

Litters of registered Beagles were obtained. Pups that had ascarides were given orally 100 mg. of piperazine* for each 5 lb. of body weight. Dogs that had hookworms were given 1 ml. of n-butyl chloride** for each 5 lb. of body weight.

Pups were given 10 ml. of anti-canine distemper and anti-infectious canine hepatitis serum† prior to the time they were shipped and after arrival in Urbana. Distemper-hepatitis vaccine‡ was administered to each pup three times during the experimental period.

Each dog was 14 weeks of age when the experiment was initiated. With one exception, assignment of the dogs was made so that littermates of the same sex were placed in each treatment group. The treatment groups were: control, nonirradiated beef (B0); beef irradiated with 2.79 (B3) megarads; and beef irradiated with 5.58 (B6) megarads. Each dog was weighed once a week. Initial body weight was considered in grouping the experimental animals. Two males and 2 females constituted each group.

The blood of each dog was examined at intervals of five weeks. Erythrocyte, leukocyte, and differential counts were made by standard clinical procedures.[1] The hemoglobin content was deter-

From the Department of Veterinary Physiology and Pharmacology, University of Illinois, and the Illinois Agricultural Experiment Station, Urbana.

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* Parlamate, produced by Parlam Corporation, New York, N.Y.

** Bu-Chlorin, produced by Pitman-Moore Co., Indianapolis, Ind.

† D-H Serum, produced by Pitman-Moore Co., Indianapolis, Ind.

‡ Virogen, produced by Pitman-Moore Co., Indianapolis, Ind.

TABLE 1—Composition of Rations

Ingredient	Total solids (%)
Tomato pomace	3.0
Soy bean meal	12.0
Wheat flakes	35.0
Dried skim milk	3.0
Sodium chloride	0.5
Salt mixture [4]	4.0
Lactalbumin	2.5
Beef (test material B0, B3, or B6)	35.0
Bone meal	2.0
Vitamin mixture*	3.0

* The following amounts of vitamins were added per 100 Gm. of ration: thiamin, 0.146 mg.; Ca pantothenate, 0.48 mg.; niacin, 2.0 mg.; riboflavin, 0.39 mg.; pyridoxine, 0.20 mg.; folic acid, 0.2 mg.; inositol, 20 mg.; para-aminobenzoic acid, 0.80 mg.; choline chloride, 273.8 mg.; menadione, 0.10 mg.; biotin, 0.166 μg.; and vitamin B_{12}, 4.8 μg.

mined by the cyanmethemoglobin method. Five milliliters of diluent was placed in a 10-ml. test tube, and 0.02 ml. of fresh blood added, the tube stoppered, and contents mixed by inversion. Readings were made on a Beckman spectrophotometer set at 540 mμ. The blank used was the diluent solution. The standard was that supplied by the National Academy of Sciences.[5] One liter of diluent solution contained 1.0 Gm. of $NaHCO_3$, 50 mg. of KCN, and 200 mg. of $K_3Fe(CN)_6$.

RESULTS AND DISCUSSION

The data on average initial body weight, average weight gained, and the feed intake per gram of gain are presented (table 2) for the 12- and 24-week periods.

504.0 Gm. per megarep of irradiation per dog. This regression of feed consumption on irradiation level was significant ($P = 0.05$). The dogs fed the 5.58-megarad irradiated beef required more feed per unit of gain than those fed the 2.79-megarad irradiated or the nonirradiated beef. The dogs ate an average of 9.3 Gm. of feed per gram of initial body weight during this 12-week period. The regression of feed consumption on initial body weight was significant ($P<0.02$).

The amount of feed consumed decreased as the level of irradiation increased during the 24-week period. This decrease in feed consumption, amounting to 995 Gm. per megarep of irradiation per dog, was significant ($P<0.05$) when adjusted for the initial weight of the dogs. The regression of feed consumption for the 24-week period was about 16 Gm. of feed per gram of initial body weight. There was no significant error regression of gain on feed intake, but this test involved only two degrees of freedom in the residual variance and so is of little importance. The dogs fed the ration containing the beef irradiated with 5.58 megarads gained an average of 4,371 Gm., those fed 2.79-megarad irradiated beef gained an average of 4,854 Gm., and those fed nonirradiated beef gained an average of 5,626 Gm. at the end of 24 weeks.

TABLE 2—Average Weight Gained and Feed Efficiency of Dogs Fed Rations Containing Nonirradiated or Irradiated Beef for 12 and 24 Weeks

Level of irradiation (megarads)	No. of dogs	Initial weight (Gm.)	Av. weight gained (Gm.)		Gm. of intake per Gm. of weight gained	
			12 weeks	24 weeks	12 weeks	24 weeks
None	4	3,993	3,710	5,626	7.71	10.22
2.79	4	3,815	3,199	4,854	7.45	10.35
5.58	4	3,872	2,902	4,371	8.47	11.50

The initial weights of the females differed significantly ($P = 0.05$) from those of the males, tending to justify stratification by sex in assignment of dogs to experimental groups. The initial body weights of the one litter of males differed significantly from those of the other litter ($P = 0.05$), tending to justify use of litters as blocks in assignment of males to experimental groups.

During the first 12-week period, both the weight gained and the feed consumed decreased as the level of irradiation of the beef was increased from 0 to 5.58 megarads. The decrease in feed consumption was

Irrespective of whether these weight gains were or were not adjusted for differences in initial body weights, there was no significant difference due to treatment. There was no significant difference in feed consumed per gram of weight gained due to treatment.

Several factors must be considered in evaluating the effects of feeding an irradiated food at a level of 35 per cent of the total solids of the ration. The contribution made by the test material toward the support of the activities of the animals would vary considerably as the test material va-

TABLE 3—Data on Blood Constituents of Beagle Dogs Fed Rations Containing Nonirradiated or Irradiated Beef for 25 Weeks

Constituents	Pre-assignment	Irradiation (megarads)		
		None	2.79	5.58
Hb (Gm./100 ml.)				
Average	14.2	18.3	17.9	17.1
Range	11.5–17.4	17.1–19.3	16.5–19.6	15.2–18.9
Erythrocytes (million/cmm.)				
Average	6.00	7.83	8.21	8.20
Range	5.16–6.97	7.06–3.60	13.00–9.18	7.59–9.26
Leukocytes (thousands/cmm.)				
Average	16.0	19.0	11.6	12.8
Range	7.6–26.3	12.5–32.1	5.5–18.1	9.6–15.4
Differential count (No./200 cells)				
Basophils				
Average	0	0	0	0
Range	0–1	0	0	0
Eosinophils				
Average	2	7	7	5
Range	0–7	4–12	5–8	3–6
Band neutrophils				
Average	5	4	8	5
Range	2–8	4–5	2–14	4–7
Segmented cells				
Average	64	49	51	57
Range	52–82	43–55	46–58	50–61
Lymphocytes				
Average	27	32	28	25
Range	14–38	25–41	24–37	21–33
Monocytes				
Average	2	4	3	2
Range	0–4	2–5	2–4	1–5
Degenerated cells				
Average	0	4	3	6
Range	0	2–8	1–6	5–7

ried from beef to flour to cabbage. The extent to which the test animal depended upon the constituents of the test material for growth, reproduction, and longevity must be taken into consideration. It is difficult to establish criteria that would be adequate for comparing all irradiated foods. However, useful comparisons can be made with calculations of the number of grams of protein, carbohydrate, or fat consumed per gram of body weight gained, and the number of calories in the test material consumed per gram of body weight gained.

The caloric content of the ration and its constituents were calculated on the basis that protein, carbohydrate, and fat contributed 4.0, 4.0, and 9.0 calories per gram, respectively. The beef in the ration supplied 50.9 per cent of the total ration calories. This amounted to an intake of 15.9 calories for each gram of body weight gained during the 24-week growth period. Therefore, in the rations containing irradiated beef, half of the calories utilized for growth came from irradiated protein or fat. The beef protein was 56.1 per cent of the total protein of the ration. The dogs ate an average of 1.10 Gm. of beef protein per gram of body weight gained. The fat in the beef constituted 86.3 per cent of the fat in the ration. The dogs ate an average of 1.27 Gm. of beef fat for each gram of body weight gained.

Table 3 shows the average and range of blood cell values of dogs prior to and at 25 weeks after assignment to the experimental groups. Abnormality was not apparent in the pre-assignment blood data. The hemoglobin values increased during the 25 weeks of the experiment. There was no difference in blood values that could be attributed to the irradiation of the beef.

SUMMARY

Nonirradiated beef or beef irradiated with 2.79 or 5.58 megarads was fed to dogs for 24 weeks. There was no significant difference in weight gains due to treatment, regardless of whether the weight gained by the dogs was or was not adjusted for differences in initial body weights. There was a significant decrease in food consumption amounting to 504 and 995 Gm. per megarep of irradiation at the end of 12 and 24 weeks, respectively. There were no significant differences in erythrocyte, leuko-

cyte, and differential counts or in hemoglobin content of the blood of dogs fed irradiated beef as compared to those fed nonirradiated beef.

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Culture and Serologic Studies on Four Dogs Inoculated with Two Leptospiral Serotypes, *Leptospira pomona* and *Leptospira canicola*

ROBERT W. MENGES, D.V.M., M.P.H.; MILDRED M. GALTON, Sc.M.;
ROBERT T. HABERMANN, D.V.M., M.S.

Atlanta, Georgia

ALTHOUGH NUMEROUS REPORTS have referred to the leptospiral carrier state in dogs, the usual method for isolation of leptospires from dog urine has been too long and laborious to undertake large surveys. As a result, most studies concerning the prevalence of leptospirosis in dogs have been serologic surveys with relatively few attempts to determine the extent of leptospiruria or to compare actual shedding with the presence of leptospiral antibodies.[1] Recently, a simple method has been developed in which urine may be obtained by bladder tap [2] and cultured directly. This method may be used for large-scale studies to detect carriers.

In the present report, the bladder tap technique was employed to obtain urine specimens for the isolation of leptospires. Prior to this study, bladder tapping of guinea pigs at necropsy and of a live canine *Leptospira canicola* carrier had proved to be successful.

The study was designed to evaluate the bladder tap technique as a method to obtain urine for isolation of leptospires by direct culture. Serum samples were obtained at the time urine was cultured so that serologic and culture results could be correlated. Histopathologic studies were done mainly to demonstrate that repeated puncture of the bladder was not harmful.

As no isolations were made from urine specimens following the first inoculum, a second inoculum was used. The two leptospiral serotypes used, *Leptospira pomona* and *L. canicola*, serve to demonstrate that infection with one serotype does not protect against infection with a second unrelated serotype.

From the Department of Health, Education and Welfare, Public Health Service, Bureau of State Services, Communicable Disease Center, Atlanta, Ga. (Menges and Galton, and the National Institutes of Health, Bethesda, Md. (Habermann).

MATERIALS AND METHODS

The 4 crossbred Pointer dogs (3 females and 1 male) used in this study were litter mates. They were 7 weeks old when obtained by the laboratory and placed in individual cages. Six days after arrival in the laboratory and again after another six-day interval, blood was tested for leptospiral antibodies and urine obtained by bladder tap was cultured. Eight days later, at the age of 2½ months, each dog was inoculated intraperitoneally with 2.0 ml. of a 33 per cent kidney suspension from *Leptospira pomona**-infected guinea pigs. Daily rectal temperatures were taken on the dogs from three days before infection until eleven days postinoculation.

After the first inoculation, blood specimens were cultured on postinoculation (PI) days 6 and 8 and urine specimens obtained by tapping the bladder were cultured on days 12, 19, 21, 26, 36, 42, and 55. The bladder was tapped with the animal suspended by its hind legs. Prior to tapping, the abdomen was thoroughly swabbed with 70 per cent alcohol. Then with a sterile, dry, 10-ml. syringe and a 3-inch, 20-gauge needle the bladder was punctured and 3 to 5 ml. of urine was withdrawn. Three drops of urine were inoculated immediately into each tube of culture medium.

On the 78th day after inoculation with *L. pomona*, the dogs were given a second inoculation (PI2) with a freshly isolated strain of *Leptospira canicola*, LT 227, at which time they were 5 months old. This culture was isolated 18 days prior to use from the urine of a 2-year-old male Boxer.**

* This strain, *L. pomona* Wickard, was isolated in 1953 by Dr. E. V. Morse from the urine of a cow. The strain had been carried by continuous passage in guinea pigs. Guinea pigs infected with this strain were received from Dr. Morse in August, 1955. The kidney suspension used to infect the dogs in this study represented the 23rd guinea pig passage in this laboratory.

** On March 4, 1957, a 6-year-old male Boxer died. The dog showed jaundice and had a *L. canicola* agglutinin titer of 1:512. The owner had another dog, a 2-year-old male Boxer, which had contact with the dog that died. This dog appeared normal on March 6, but the owner said that it had been off feed for one day on February 20, 1957. The blood serum agglutinin titer against *L. canicola* on March 6th was 1:4,096. The same day, urine obtained by tapping the bladder was inoculated into Fletcher's semisolid medium. Leptospires were observed in the urine on dark field examination, and appeared in the cultures the fifth day after inoculation. The leptospires were identified as *L. canicola* and referred to as "strain LT 227."

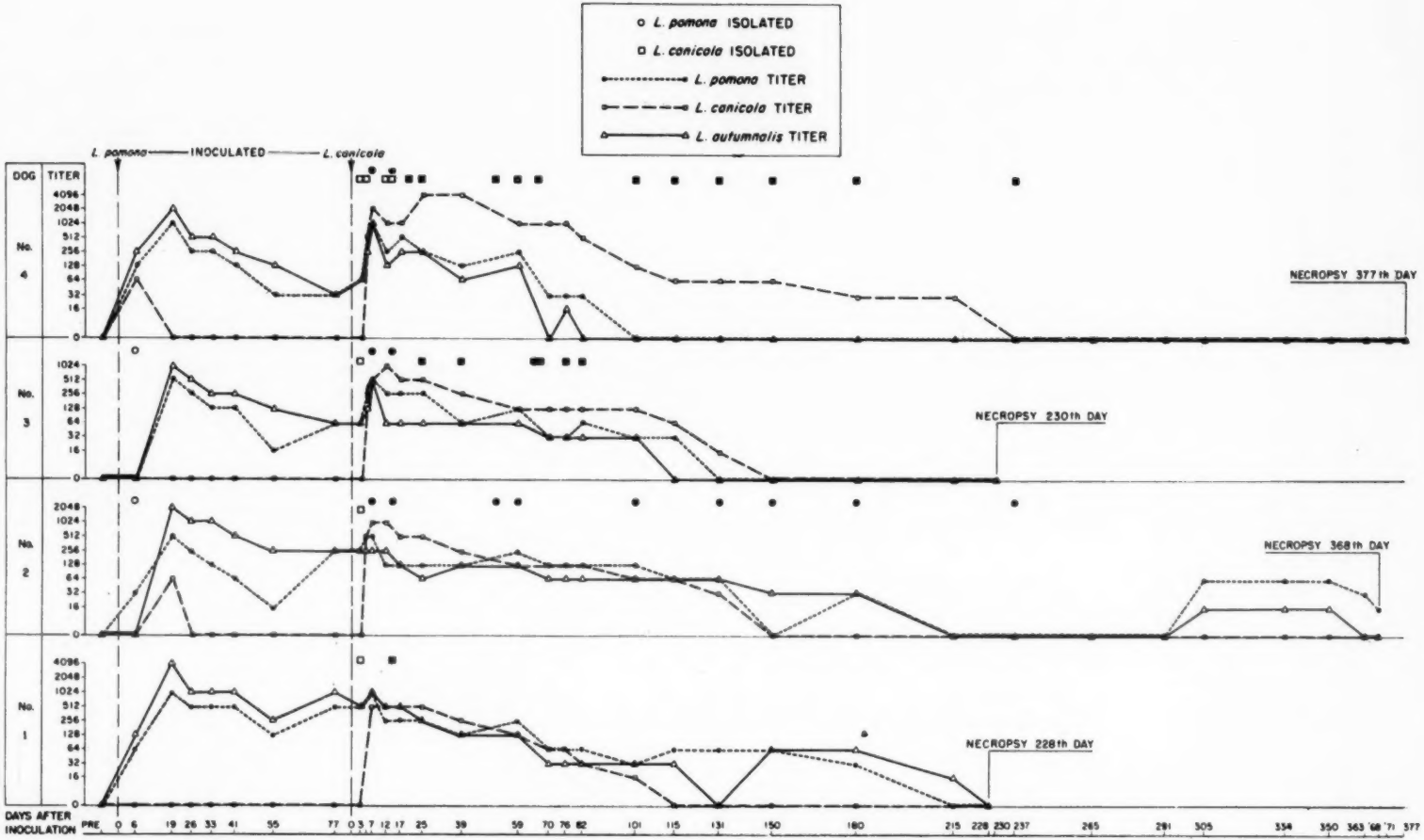
L. pomona ISOLATED
L. canicola ISOLATED
L. pomona TITER
L. canicola TITER
L. autumnalis TITER
L. pomona
INOCULATED
L. canicola
DOG
TITER
No. 4
No. 3
No. 2
No. 1
NECROPSY 377th DAY
NECROPSY 230th DAY
NECROPSY 368th DAY
NECROPSY 228th DAY
DAYS AFTER INOCULATION
PRE 0 6 19 26 33 41 55 77 0 3 7 12 17 25 39 59 70 76 82 101 115 131 150 180 215 228 230 237 265 291 305 354 350 363 '68 '71 377

Fig. 1—Serologic and culture results on 4 dogs experimentally inoculated with Leptospira pomona and Leptospira canicola. White circles and squares indicate that the isolations were made from blood; black circles and squares indicate that isolations were made from urine.

One-ml. portions of the 23-day-old original cultures in Fletcher's semisolid medium were inoculated intraperitoneally into each of the 4 dogs.

Rectal temperatures were taken on PI2 days 3 to 14, following the second inoculation, and blood and urine specimens were cultured. Blood specimens were cultured on PI2 days 3, 5, 7, 10, 12, and 14. Urine specimens obtained by bladder tap were cultured on PI2 days 7, 14, 21, 25, 39, 42, 59, 66, 67, 76, 77, 82, 83, 101, 115, 116, 117, 131, 150, 180, 215, 237, 265, 291, 305, 308, 334, 343, 346, 350, 355, 356, 363, 364, 368, 369, 371, and 377.

Each specimen obtained from the dogs was cultured in six tubes of medium. For the first seven months, each specimen was inoculated into three tubes of Fletcher's semisolid medium and three tubes of Chang's semisolid medium. After this period, specimens were inoculated into six tubes of Fletcher's semisolid medium. Three drops of blood or urine were inoculated into each tube of medium.

After inoculation, all cultures were incubated at 28 to 30 C. and held for a minimum of one month before being discarded. Dark field examinations were made at one to two weeks and at four weeks. When leptospires were found, the positive tubes were subcultured several times at five- to ten-day intervals in Stuart's medium. Five-day-old cultures were tested against *L. pomona* and *L. canicola* hyperimmune rabbit serums by the microscopic agglutination test.

At approximately the same time that specimens were cultured, blood was obtained for serologic tests. The serums were tested by the microscopic agglutination test with formalinized Leptospira antigens of *L. autumnalis*, AB, Akiyomi A; *L. ballum*, S102; *L. canicola*, Ruebush; *L. icterohaemorrhagiae*, AB, Wijnberg; *L. pomona*, S91; and *L. sejroe*, Mallersdorf.

The dogs were killed and necropsied after at least five consecutive urine cultures from each animal appeared negative. Portions of all organs were placed in 10 per cent formalin solution for histopathologic studies. Silver staining of the tissues was done using the Warthin-Starry technique.[3] At necropsy, eye fluid (aqueous and vitreous humor), urine, and a 10 per cent suspension of kidney tissue were cultured.

LABORATORY FINDINGS

The culture and serologic findings on these four dogs are summarized (table 1; fig. 1).

Dog No. 1.—No leptospires were isolated from the blood or urine samples from this dog after the first inoculation. Leptospiral antibodies were detected on PI day 6 in serum dilutions of 1:64 and 1:128 against *L. pomona* and *L. autumnalis* antigens, respectively. A rise in titer to 1:1,024 to *L. pomona* and 1:4,096 to *L. autumnalis* was observed on day 19. Thereafter, the titers showed a four- and twofold drop to the respective antigens and remained at that level for 41 days. It is notable that the reactions with *L. autumnalis* were consistently higher than with *L. pomona*.

L. canicola was isolated from the blood on PI2 day 3 and from the urine only on PI2 day 14. All other blood and urine cultures were negative. Antibodies against *L. canicola* developed rapidly with a peak titer of 1:512 on PI2 day 7. This titer persisted until day 25, then gradually dropped to less than 1:16 by day 115. During the first week, titers against *L. pomona* and *L. autumnalis* showed a slight rise.

Necropsy.—This dog was necropsied at 1 year of age, 306 days after the first inoculation (PI2 day 228). The gross lesions consisted of two small, dark red areas in the lungs 1/8 inch in diameter and dark purple areas at the tip of the spleen. The microscopic findings were as follows. The spleen showed good follicle formation, but densely staining basophilic cells were in moderate numbers. There were moderate quantities of red pulp and a considerable quantity of yellowish-brown pigment in the phagocytes. The liver showed hyperemia and an occasional microscopic nodule of lymphocytes. Although the urinary bladder was punctured 75 times, it showed no gross or microscopic lesions. The kidney showed slight hyperemia and slight thickening of an occasional Bowman's capsule. The nearby arterioles were prominent and showed some thickening of the media. The interstitial tissue in these areas showed thickening with bands of fibrous connective tissue. The medulla of the kidney showed several lesions about 150 to 200 μ in diameter with fibrosis and infiltration of lymphocytes. Some of the early lesions showed proliferation of tubular epithelial cells and fibroblasts. Later lesions showed dense bands of fibrous connective tissue infiltrated with lymphocytes and often involving several tubules. Silver stain on liver and kidney showed no leptospires.

Dog No. 2.—Leptospira pomona was isolated from the blood of this dog on PI day 6, but repeated urine cultures did not yield this type until seven days after the second inoculation or 85 days after the first inoculation. *Leptospira canicola* was recovered only from the blood on PI2 day 3. Between PI2 days 7 and 237, *L. pomona* was isolated intermittently from nine to 17 urine samples cultured. Thus the dog was shedding *L. pomona* on the 315th day after inocula-

tion with this type. On PI day 6, antibodies were detected only in a serum dilution of 1:32 against *L. pomona* antigen. However, by day 19 the titer rose to 1:512 against *L. pomona* and 1:2,048 to *L. autumnalis*. After the *L. canicola* inoculation, antibodies for this type developed rapidly to 1:512 by PI2 day 5 and to the same level against *L. icterohaemorrhagiae*. The titer against *L. canicola* never exceeded that against *L. icterohaemorrhagiae* until after PI2 day 115. No antibodies could be detected against *L. canicola* after PI2 day 131. Although this dog continued to shed *L. pomona* through PI2 day 315, the titer to this type dropped to 1:64 on PI2 day 131 and varied between this level and no reaction in a 1:16 dilution until necropsied.

Necropsy.—The dog was necropsied at 1 year and 5 months of age, 446 days after the first inoculation (PI2 day 368). The gross lesions consisted of dark red to black areas throughout the lungs, enlarged bronchial lymph nodes, and a thickened spleen which was dark purple. The microscopic findings were as follows. The lung showed scattered, small areas in which the bronchioles and nearby alveoli were distended with serosanguineous fluid. There was also an occasional bronchiole showing peribronchial infiltration of lymphocytes and monocytes and a few polymorphonuclear leukocytes and eosinophils. The spleen showed good follicle formation, densely staining basophilic cells in moderate numbers, red pulp in large quantity, and some yellowish-brown pigment in the phagocytes. The kidneys showed no significant lesions. The urinary bladder was punctured 61 times but had no significant lesions. In one area, several of the small vessels were distended with blood. The uterus showed some enlargement of the uterine glands and proliferation of the endometrium into the lumen. Silver-stained kidney sections revealed no leptospires.

Dog No. 3.—Leptospira pomona was isolated from the blood of this dog on the sixth day after inoculation but, as with dog 2, direct cultures of six urine samples obtained on PI day 19 to 55 yielded no leptospires. On PI2 day 3, *L. canicola* was isolated from the blood. Then, on PI2 days 7 and 14, urine cultures revealed *L. pomona*. This was 85 and 92 days, respectively, after the dog was inoculated with *L. pomona*. The next six urine samples cultured between PI2 days 25 and 82 yielded *L. canicola*. All subsequent urine cultures were negative.

Serologic findings on this dog followed much the same pattern as in dogs 1 and 2, except that no antibodies were detected on the sixth day after the first inoculation, when *L. pomona* was isolated from the blood. The peak agglutinin titers against *L. pomona* and *L. autumnalis* occurred on PI day 19, followed by a gradual decrease. After the second inoculation, the titer to *L. canicola* rose to a peak by the twelfth day, and during this time a significant rise was noticed in the titers to *L. pomona* and *L. autumnalis*. Culture and serologic findings were negative when the dog was necropsied 308 days after the first experimental inoculation (PI2 day 230).

Necropsy.—The gross lesions consisted only of dark purple areas at the tip of the spleen. The microscopic findings were as follows. The liver showed slight hyperemia. The spleen showed follicle formation, but densely staining basophilic cells were in moderate numbers. There was a moderate quantity of red pulp and some pigment in the phagocytes. The kidneys showed hyperemia, and an occasional Bowman's capsule was thickened with fibrous connective tissue.

There was also an occasional tubule that showed slight interstitial fibrosis. Silver staining of kidney sections showed no leptospires. Although the urinary bladder was punctured 43 times, it did not show any significant lesions.

Dog No. 4.—Leptospira pomona was not isolated from the blood of this dog nor was it obtained from the urine until seven days after inoculation with *L. canicola* (85 days after initial inoculation). Blood cultures yielded *L. canicola* on PI2 days 3, 5, 12, and 14. This type then appeared in the urine twelve times on PI2 days 14 to 237. Subsequent urine cultures through the 377th day were negative.

Again, in this dog, the agglutinin titers to *L. autumnalis* were consistently higher than to *L. pomona*. The peak titers occurred on PI day 19, with gradual decrease to 1:32 to both antigens on PI day 77. A second rise occurred during the first week following inoculation with *L. canicola*. It is interesting that this dog was serologically negative to *L. canicola* on PI2 day 237, when this type was last isolated from the urine.

Necropsy.—This dog was necropsied at 1 year and 5 months of age, 455 days after the first inoculation (PI2 day 377). The gross lesions consisted of a yellowish, mot-

tled appearance of the anterior lobe of the left lung. The microscopic findings were as follows. The lung showed scattered, small areas of perivascular and peribronchial infiltration of lymphocytes, monocytes, and plasma cells; an occasional clump of macrophages was seen in the alveoli. The spleen showed follicle formation and densely staining basophilic cells were not numerous. The liver showed hyperemia. The kidney showed an occasional Bowman's capsule that was thickened, and an occasional glomerulus was seen that had protein-like material between the glomerular tuft and Bowman's capsule. The urinary bladder was punctured 69 times and did not show significant lesions. A cervical lymph node had a small abscess about 100 μ in diameter, containing polymorphonuclear leukocytes, and was surrounded by strands of fibrous connective tissue. Another lymph node had a small granulomatous lesion, about 100 μ in diameter, made up of large mononuclear cells with some fibroblasts. The center of the lesion contained a round, hyaline, protein-like mass.

months. After these periods, repeated urine cultures were negative on these dogs. At necropsy, no leptospires were isolated from kidney suspensions or demonstrated in stained sections of the kidney tissue, which indicates that they were free of infection.

The experiment also demonstrated that uncontaminated urine specimens are not always obtained. Following the first inoculation, 13 (48%) of the urine specimens were contaminated; after the second inoculation, 12 (15%) were contaminated. This suggests improvement in technique as the experiment progressed. Some contamination can be avoided by thoroughly cleansing the skin and then swabbing with 70 per cent alcohol prior to tapping. However, if bacteria are present in the bladder, urine contamination cannot be prevented.

Following the first inoculation, no leptospires were isolated from the urine specimens. This may have been due, in part, to contamination; however, it would not account for the failure to isolate leptospires from the 14 (52%) urine specimens which showed no growth. Obviously, 3 of the dogs

TABLE 1—Summary of the Culture Results on 31 Blood Specimens and 107 Urine Specimens Obtained from Four Experimentally Infected Dogs

Dog No.	Blood			Urine		
	Total No. specimens cultured	No. contaminated specimens	No. positive	Total No. specimens cultured	No. contaminated specimens	No. positive
FIRST INOCULUM						
1	2	0	0	8	5(63%)	0
2	2	0	1(50%)	6	1(17%)	0
3	2	0	1(50%)	6	3(50%)	0
4	2	0	0	7	4(56%)	0
Totals	8	0	2(25%)	27	13(48%)	0
SECOND INOCULUM						
1	6	0	1(17%)	10	1(10%)	1(11%)*
2	6	0	1(17%)	26	3(12%)	9(39%)
3	6	0	1(17%)	15		8(53%)
4	5	0	4(80%)	29	8(28%)	13(63%)
Totals	23	0	7(30%)	80	12(15%)	31(46%)

* Percentage of uncontaminated cultures.

At necropsy, eye fluid (aqueous and vitreous humor), urine, and a 10 per cent suspension of kidney tissue were cultured for each dog. No leptospires were isolated from any of the tissues cultured.

DISCUSSION

The value of the bladder-tap technique for isolation of leptospires from urine by direct culture was demonstrated in this experiment. Dog 2 proved to be a carrier of *L. pomona* for 10½ months and dog 4 was found to shed *L. canicola* for eight

were harboring *L. pomona* during this period, as this type was isolated from their urine seven days after they were inoculated with *L. canicola*. This suggests that the second inoculation stimulated shedding of the first serotype. The possibility of intermittent shedding must be considered. In addition, recent work with experimentally infected guinea pigs has indicated that more isolations are obtained with smaller inoculums of urine, suggesting that urine may be toxic for leptospires. Evidence indicates that the urine should be

diluted with buffered sodium chloride and 1 drop of each dilution cultured. More isolations have been made with diluted urine than with undiluted urine, and dilution of the urine appears to decrease contamination. Leptospires have been isolated from urine specimens diluted 1:100,000, or a final dilution of 1:15,300,000 in a tube containing 5.0 ml. of Fletcher's semisolid medium.

The bladder-tap technique has been used on field cases, and some veterinarians have asked if repeated puncture of the bladder is harmful to the animal. During this study, the bladders of the experimentally infected dogs were punctured repeatedly for 15 months and histopathologic studies of each bladder showed no harmful effects. In actual practice it is necessary to tap the bladder only once or twice for detection of infected animals or carriers.

The fact that 1 dog was serologically negative when leptospires were isolated from the blood six days after inoculation and another showed no agglutinins when leptospires were recovered from urine on the 237th day, emphasizes the limitations of antibody detection as a method of confirming infection both during the early stages or the late carrier period. The serologic patterns developed in these 4 dogs also illustrate the limitation of antibody response as a means of determining infecting serotype.

SUMMARY

The bladder-tap technique was tested on 4 dogs experimentally infected with *Leptospira pomona* and *Leptospira canicola*. The dogs were inoculated first with guinea pig kidney tissue suspension containing *L. pomona* (Wickard strain); 78 days later they were inoculated with 1.0-ml. portions of a culture of *L. canicola* (strain LT 227). Following the *L. pomona* inoculation, this serotype was isolated from two (25%) of eight blood specimens but no isolations were made from 27 urine specimens; 48 per cent of these urine specimens were contaminated. Following the *L. canicola* inoculation, this type was isolated from seven (30%) of 23 blood specimens. Leptospires were isolated from 31 (39%) of 80 urine specimens cultured. Of these, 12 were *L. pomona* and 19 were *L. canicola;* 12 (15%) of the 80 urine specimens were contaminated.

Histopathologic studies demonstrated that repeated puncture of the bladder was not harmful. The experiment demonstrated that infection with one serotype did not protect against infection with a second unrelated serotype. In addition, the study clearly showed that the infecting serotype cannot be determined by antibody response.

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Studies on a Pox Disease of Monkeys. I. Pathology

R. M. SAUER, V.M.D.; J. E. PRIER, D.V.M., Ph.D.; R. S. BUCHANAN, V.M.D.;
A. A. CREAMER, V.M.D.; H. C. FEGLEY, V.M.D.

Philadelphia and West Point, Pennsylvania

A POX DISEASE was observed in a colony of 2,000 captive monkeys during February, 1959. Pox lesions were never observed prior to this epizootic, even though the colony had been maintained for several years under close supervision and all new monkeys were handled individually before admission.

This colony was composed of *Macaca philippinensis* (41%), *Macaca mulatta* (56%), and *Cercopithecus aethiops* var. *sabaeus* (3%) housed primarily in large gang cages. Buildings containing the colony consisted of five large rooms, each with three to six gang cages. Although the rooms were separated completely from one another, isolation was incomplete. Groups of monkeys were moved occasionally from one room to another and were under the care of the same handlers.

The first pox lesions were noticed in two groups of monkeys that had been in the colony for some time and were exposed to several new shipments of animals. Clinical signs of infection appeared sporadically in other parts of the colony and small numbers of all ages and both sexes were affected clinically.

Because of the large numbers of animals in a given cage and the inability to examine each animal, it is impossible to state the total number of animals showing clinical evidence of disease. At the time of the epizootic, the impression was that perhaps only a dozen animals or so were involved, but, in retrospect, it may be that as many as 10 per cent of the colony had the disease. Of those animals showing observable physical indications of infection, a high percentage were *M. philippinensis*. Only a few *M. mulatta* exhibited clinical pox lesions. In one group of 19 *M. philippinensis*, 6 showed clinical lesions and the serums of 18 showed titers against the specific antigen.[8] Clinical evidence of infection was observed in all of a group of 6 nursing infants; 1 died. The total number of deaths due to pox disease in the colony was less than 0.5 per cent.

Fig. 1—Edema of the face in a monkey with pox infection.

This report describes the pathologic changes of a pox disease of monkeys.

GROSS PATHOLOGIC FINDINGS

In most cases, no signs of illness were noticed prior to the appearance of cutaneous lesions; however, a few individuals showed edema of the face which began over

Fig. 2—Discrete cutaneous lesions on the hand of a pox-infected monkey.

From the Laboratory of Pathology (Sauer) and the Section in Medicine (Prier), School of Veterinary Medicine, University of Pennsylvania, Philadelphia; and the Biological Development & Control Laboratories (Prier and Fegley) and Veterinary Department (Buchanan and Creamer), Merck Sharp & Dohme, Division of Merck & Co., Inc., West Point, Pa.

Fig. 3—Histopathologic section of skin shows acanthosis with formation of broad, elongated rete ridges. H & E stain; x 7.

Fig. 4—Histopathologic section of epidermis shows (a) marked intracellular edema, and (b) necrosis. H & E stain; x 75.

the bridge of the nose and extended laterally to involve the eyelids. In some instances, this edema caused complete closure of the affected eyes (fig. 1).

Cutaneous manifestation occurred as a single crop and consisted of multiple, discrete, blanched, shot-like papules varying in diameter from less than 1 mm. to approximately 4 mm. The papules were either pointed or somewhat flattened. Many showed dark reddish-brown umbilicated centers (fig. 2). The surface of some of the non-umbilicated lesions appeared delicate and could be removed easily by the slightest trauma, leaving a faintly moist surface. Expression of a pustule revealed thick, creamy, grayish, purulent material. Later these became covered with reddish-brown crusts which fell off in seven to ten days, leaving a small scar.

Eruptions appeared on any portion of the cutaneous surface; lateral and dorsal aspects of the tongue; oral, pharyngeal, laryngeal, and tracheal mucosae, but were most commonly observed on the buttocks, hands, feet, and hind limbs. The papules on the extensor surfaces of the feet were usually the largest and most definitely umbilicated.

Oral lesions, when present, were always ulcerated. The ulcers were circular, discrete, and averaged 2 mm. in diameter. They were crater-like with thick, grayish, opaque margins and shallow, grayish-tan centers.

The eruptions in infants and in adults with fatal cases had a tendency to become hemorrhagic.

The only visceral lesions found consistently in all monkeys with fatal cases and in affected euthanatized animals were foci of necrosis in lymphatic tissue; *i.e.*, spleen, lymph nodes, and solitary follicles of the digestive tract. At necropsy, 1 individual showed widespread petechial and ecchymotic hemorrhages involving particularly the skin, subcutaneous fat, peritoneum, omentum, lungs, and gastrointestinal mucosa.

HISTOPATHOLOGIC FINDINGS

The microscopic appearance of the skin was characterized by proliferation of the epidermis followed by necrosis.

In early lesions, the prominent feature was a focal area of acanthosis, often with

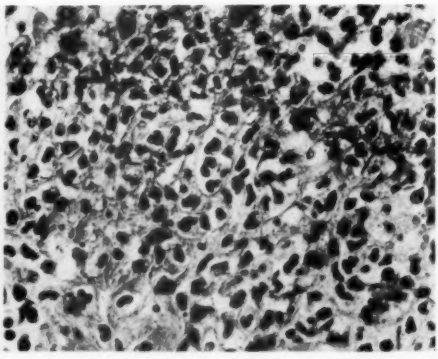

Fig. 5—Histopathologic section of skin shows spongiform pustule. Destruction of cell nuclei and cytoplasm but not of cell walls is visible. H & E stain; x 326.

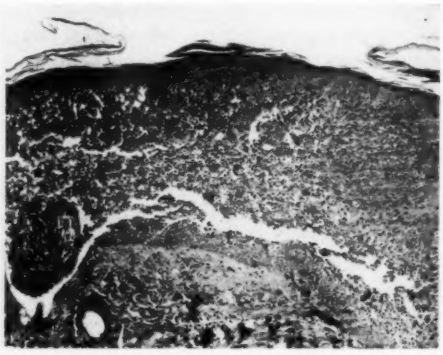

Fig. 6—Histopathologic section shows severe necrosis of entire epidermis. H & E stain; x 59.

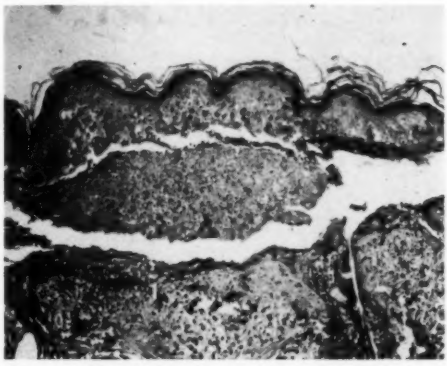

Fig. 7—Histopathologic section shows necrosis of lower two thirds of epidermis. H & E stain; x 75.

the formation of broad, elongated, rete ridges (fig. 3). The thickness of the epidermis was increased from the usual three to four layers of cells to 25 or 30 layers. Intracellular edema produced a great increase in the size of the cell body and nucleus (fig. 4). The nucleoli were large, often eosinophilic, and were frequently paired. Intracellular edema rarely proceeded to reticular degeneration with the formation of a large vesicle before the onset of necrosis. If reticular degeneration occurred, the areas were usually found in the upper half of the epidermis.

Inflammatory cells accumulated in the perivascular regions of the dermis. Neutrophils invaded the edematous squamous cells with consequent destruction of the cytoplasm and nucleus but not of the cellular walls (fig. 5). This resulted in a spongiform pustule.[6] Rupture of the interstices to form a large confluent pustule was not commonly seen; however, multiple small pustules were frequent.

The formation of characteristic intraepidermal vesicles as described in lesions of variola [1,5,7,10] was overshadowed, in most instances, by coagulation necrosis. As stated earlier, large confluent vesicles and pustules, although seen occasionally, were infrequent, for coagulation necrosis occurred early and began any time following the intracellular edema. Often the entire epidermis appeared as an eosinophilic mass containing ghosts of epidermal cells, scattered pyknotic nuclei, and a few neutrophils (fig. 6). At times the necrosis was zonal, being primarily low, intermediate, or high in the epidermis (fig. 7). A large pustule often formed by liquefaction of the debris following invasion of the necrotic mass by polymorphonuclear leukocytes.

Intracytoplasmic inclusion bodies were most numerous in epidermal cells along the sides of the lesion (fig. 8), but also occurred at the base. These usually were round but frequently were somewhat irregular, eosinophilic, and approximately 3 to 7μ in diameter. They were singular or multiple, with as many as four small bodies seen in one cell, and usually were surrounded by a clear halo. Eosinophilic intranuclear inclusions occasionally were seen (fig. 9), but never concurrently in a cell with intracytoplasmic inclusion bodies.

The microscopic appearance of lesions of the gastrointestinal tract was characterized by focal areas of necrosis in the mucosa.

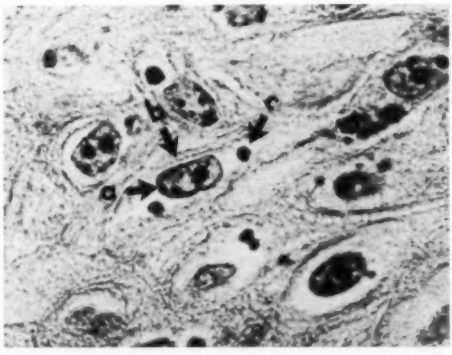

Fig. 8—Microscopic examination of epidermal cells shows intracytoplasmic inclusion bodies: (a) nucleus, (b) cell wall, and (c) intracytoplasmic inclusion. H & E stain; x 759.

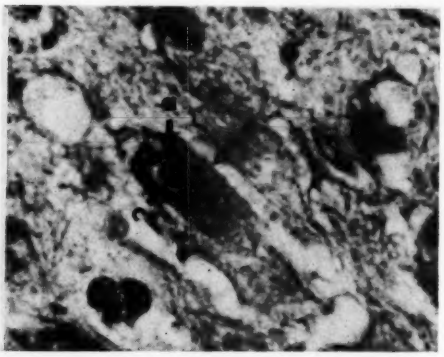

Fig. 9—Microscopic examination of epidermal cells shows intranuclear inclusion body: (a) nucleus, (b) cell wall, and (c) intranuclear inclusion. H & E stain; x 1333.

Epithelial cells in and near the lesions possessed large, frequently paired nucleoli. Some epithelial cells contained large homogeneous, eosinophilic bodies within their nuclei, which suggested an intranuclear inclusion. Necrosis of entire solitary lymph follicles was common.

DISCUSSION

A review of the literature on pox diseases in monkeys reveals that a spontaneous outbreak of pox in captive monkeys has not been reported until 1959 [12]; however, a number of investigators have experimentally transmitted variola virus to *M. philippinensis, M. mulatta, M. fuscatus,* and *Cercopithecus.*[11] Wild Myeetes sp. and Cebus sp. have been reportedly involved in a variola epidemic which occurred among natives from the region of the upper Uruguay River in Brazil.[2] The orangutan also was found to be susceptible to infection by variola virus.[3] A description of a papular disease of unknown etiology has been reported in an orangutan *(Pongo pygmaeus).*[4] A virus in the variola-vaccinia group was found to be the etiologic agent of a pox disease in *M. philippinensis.*[12]

The umbilicated cutaneous lesions seen in the February, 1959 pox disease of monkeys were not unlike those reported for human beings with cases of variola. Cytoplasmic inclusion bodies are common to viruses of the variola-vaccinia group and the pox disease of monkeys. Vaccinia and the etiologic agents of pox diseases in other animals do not produce intranuclear bodies such as those seen in variola and pox of monkeys. Work is now under way to ascertain the relationship of this agent to other members of the pox group.

SUMMARY

A pox disease was observed in a large colony of *Macaca philippinensis, Macaca mulatta,* and *Cercopithecus aethiops* var. *sabaeus.* The gross and microscopic pathology of tissues from naturally affected animals is described. The most significant finding was the presence of intranuclear and intracytoplasmic epidermal inclusion bodies. The relationship of this disease to other pox diseases is discussed.

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Studies on a Pox Disease of Monkeys. II. Isolation of the Etiologic Agent

J. E. PRIER, D.V.M., Ph.D.; R. M. SAUER, V.M.D.; R. G. MALSBERGER, Ph.D.; J. M. SILLAMAN, B.S.

West Point and Philadelphia, Pennsylvania

THIS REPORT concerns the isolation and partial description of a viral agent from active cases of a disease of monkeys characterized by a generalized pox-like skin eruption, low mortality, high morbidity, and specific pathologic features.

MATERIALS AND METHODS

Tissue Culture.—Rabbit and monkey kidney cell monolayer sheets were used to culture the agent. Rabbit kidneys were treated with 0.25 per cent trypsin at room temperature for two to three hours and monkey kidneys for 16 hours at 5 C. In both, the tissues were agitated during the trypsinization period with a magnetic stirrer. Rabbit kidney cells were grown in medium 199 with 10 per cent calf serum, and maintained and seeded in medium 199 with 2 per cent calf serum. The latter medium was used for both growing and seeding monkey kidney cells. All cell sheets were screened microscopically for the presence of contaminating viral agents before use. Both roller tubes and 1-liter Blake bottles were used for cell propagation. Cells were inoculated after six to ten days of growth, depending on the microscopic appearance of the monolayer sheet, and fluids were harvested when cytopathogenic effects were evident in the majority of the cells.

Chicken Embryos.—The agent was inoculated in 0.2-ml. amounts on the chorioallantoic membranes of 10- or 12-day chicken embryos. All membranes were examined approximately 72 hours after injection.

Isolation Procedure.—A *Macaca philippinensis* died with symptoms of pox complicated by severe generalized edema and respiratory failure.[1] At autopsy, there were extensive necrotic areas in the mouth involving the gingival and buccal mucosae.

Sections of a lesion on the buccal mucosa were removed and ground in a mortar with a sterile abrasive* and with medium 199 as a diluent. Supernatant fluid (0.5 ml.) was inoculated into each roller tube of primary rabbit kidney cells. Dry swabs were used to scrape both mouth and skin lesions and were then placed in 4.0 ml. of Hanks' balanced salt solution. Heart blood was used for isolation by placing several drops in brain-heart infusion broth (Difco). The same volume of fluid was used to inoculate roller tubes in all cases.

TABLE 1—Number of M. philippinensis Monkeys (Naturally Exposed to a Pox-like Agent) Showing Various Pre- and Postexposure Serum Titers

Exposure	Hemagglutination-inhibition titer				
	<10	10	40	640	>2,160
Pre-exposure	16	1	1	..	..
21 days after exposure	1	..	..	1	16

Hemagglutination Tests.—Infected chicken embryo chorioallantoic membranes were used as a source of hemagglutinin. Groups of membranes were ground with the sterile abrasive in a mortar and 2 ml. of sodium chloride (0.9%) was added per membrane. The resulting homogeneous mixture was clarified by low-speed centrifugation. The supernatant fluid was removed, distributed into ampules, and held at −60 C. To determine hemagglutinin titers, serial twofold dilutions were made in sodium chloride and 0.5 ml. of each dilution was mixed with 0.2 ml. of a 1 per cent suspension of chicken erythrocytes. In hemagglutination-inhibition tests, 0.25 ml. of a virus dilution containing 4 hemagglutinating units was mixed with an equal amount of a serum dilution and incubated for one hour before adding 0.2 ml. of the erythrocyte suspension. The serum titer was expressed as the reciprocal of the highest dilution of serum completely inhibiting agglutination.

RESULTS

Initial isolation of virus was attempted on roller tubes of rabbit kidney cells. Forty-

TABLE 2—Complement-Fixation Test Titers Following Immunization with Vaccinia and "Monkey Pox" Viruses

Rabbit No.	Immunizing virus	Titers	
		Vaccinia antigen	"Monkey pox" antigen
381	Vaccinia	1:32	1:8
382	Vaccinia	1:96	1:8
383	"Monkey pox"	1:24	1:96
384	"Monkey pox"	1:8	1:64
331	"Monkey pox"	1:32	1:192
332	"Monkey pox"	1:32	1:256
Control	.....	Neg.	Neg.

From the Biological Development and Control Laboratories, Merck Sharp & Dohme, West Point, Pa. (Prier, Malsberger, Sillaman); and the Section in Medicine (Prier) and the Laboratory of Pathology (Sauer), School of Veterinary Medicine, University of Pennsylvania, Philadelphia.

Serologic tests were performed by Miss Joan Davitt, of the Biological Control Laboratory, Merck Sharp & Dohme.

* Alundum, produced by the Norton Co., Worcester, Mass.

eight hours after inoculation, the cells showed extensive cytopathologic changes, and by 72 hours the sheets had sloughed from the glass. The fluid and cell debris were harvested and a second passage was made in monkey kidney cells. After 48 hours, cytopathic changes were evident and cells had begun to slough from the glass.

Preparations were stained with Giemsa stain and microscopic examination for cytopathogenic details was made. Islands of severely affected cells were present throughout the sheets. Cells were shrunken with some cytoplasmic fibril formation. The most notable lesion was the presence of large intracytoplasmic eosinophilic inclusions (fig. 1). These bodies were similar to those previously seen in the microscopic skin lesions of affected monkeys.[1] Occasional intranuclear inclusions also were seen. The bodies varied in shape, size, and location; were single or multiple; and frequently were surrounded by a clear halo. Although the bodies cannot be unequivocally labeled as Guarnieri's bodies, the resemblance to variola-vaccinia inclusions is clear.

Tissue culture fluids of the first rabbit kidney cell passage were placed, undiluted, on the chorioallantoic membranes of embryonating chicken eggs. After 72 hours, the membranes were harvested and examined. All showed severe edema, confluent raised areas, and small, isolated, irregularly shaped areas at the periphery of the membrane. The lesions were smaller, less raised, and showed no hemorrhagic tendencies, as compared to eggs inoculated with vaccinia virus. A titer of 10^{-4} was obtained in eggs on passage of tissue culture fluid. The pathogenicity of the virus for the chorioallantoic membrane was neutralized by pox antiserum (monkey origin).

Suspensions of chorioallantoic membrane infected with the pox agent were examined in serial twofold dilutions for the presence of hemagglutinin for chicken erythrocytes. Employing serial twofold dilutions, a titer of 128 was obtained. The hemagglutination was inhibited by serum obtained from monkeys that had recovered from the infection. Cross hemagglutination-inhibition tests with vaccinia and "monkey pox" antiserums showed that there was no difference between the hemagglutinin of the 2 viruses. Erythrocytes from the following species were not agglutinated by the pox virus: ox, horse, dog, cat, man (type "O"), guinea pig, hamster, mouse, *M. mulatta, M. philippinensis,* and sheep.

Paired serums from *M. philippinensis* naturally exposed to the disease were tested (table 1). Serums from 2 monkeys of a total of 18 showed a low titer in the initial sample, and all but 1 showed high titers in the postinfection sample, indicating almost complete infection of the group. Only 6 animals of the group, however, showed definite clinical signs. A group of 23 *M. mulatta* that were in the same area during the infection period were tested for hemagglutinin inhibition antibodies with positive results in serums from 6 monkeys.

Complement-fixation tests using a 50 per cent end point titration with both vaccinia

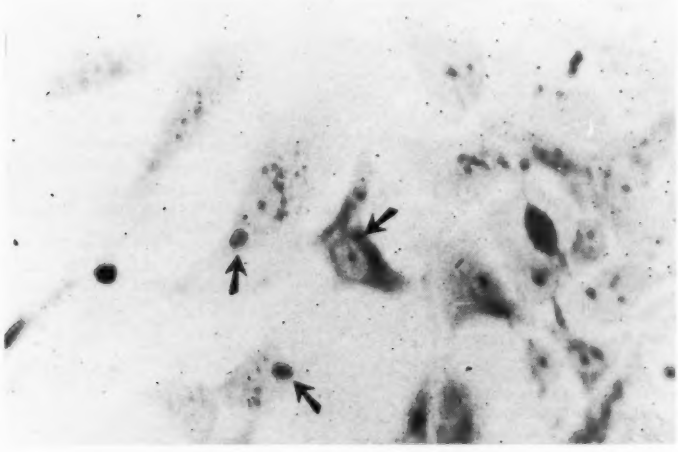

Fig. 1—Intracytoplasmic inclusion bodies in monkey kidney epithelial cells infected with "monkey pox" virus, strain 1744; x 540.

TABLE 3—Pathogenicity of "Monkey Pox" Virus for Laboratory Animals

Animal	Route of infection	Results of infection
Rabbit	Skin scarification	Local vesiculation; secondary generalized pox without evidence of systemic disease
Rabbit	Subcutaneous	Local induration and ulceration; secondary generalized pox
Guinea pig	Intradermal	Negative
Guinea pig	Foot pad	Local swelling and induration
Mouse	Intraperitoneal	Negative
Mouse	Intracerebral	Death; 6 passages
M. mulatta	Intravenous	Systemic pox
M. mulatta	Skin scarification	Local lesion
M. philippinensis	Skin scarification	Local lesion

and "monkey pox" viruses against their homologous and heterologous serums were performed (table 2). In each case, the homologous system yielded a higher titer, indicating some antigenic difference between the two agents. Proper dilutions of reagents were determined by box-type titrations.

A suspension of material scraped from monkey skin lesions was placed on the scarified skin of 2 rabbits. After six days, raised vesicular lesions appeared in the inoculated area. Nine days after inoculation, 1 animal developed a generalized pox-type eruption (fig. 2). The virus has been passed in rabbits intradermally for three successful passages.

Subcutaneous injection of pox virus in rabbits resulted in a large ulcerated area at the point of injection with secondary generalized pox eruptions covering many areas of the body.

Following injection into the foot pads of guinea pigs, a local, enlarged, granuloma-like lesion appeared with no evidence of systemic disease. Intradermal injection or subcutaneous injection in an area over the abdomen did not produce positive reactions.

Intraperitoneal injections in 3-week-old mice did not produce detectable disease. Intracerebral injections in 3- to 6-week-old mice resulted in fatal encephalitis. The virus has been carried through six intracerebral passages in mice with uniform deaths occurring in all passages (table 3).

Tissue culture fluid of the second virus passage was used as the inoculum in all cases to initiate infections in animals. The infectivity by intracerebral inoculation of mice was neutralized by a 1:10 dilution of anti-pox serum.

Since the original isolation, virus has been recovered from several additional *M. philippinensis* and a naturally infected *M. mulatta*. Virus also has been isolated from an experimentally infected *M. mulatta* that

Fig. 2—Multiple pox lesions on rabbit skin following scarification.

developed distinct lesions that were considered typical of the pox-like disease in monkeys. The virus is also pathogenic for monkeys when given by the skin scarification technique. These studies will be discussed in a later report.

SUMMARY AND CONCLUSIONS

A viral agent from a pox-like disease in monkeys has been isolated. The virus was propagated in tissue culture and on the chorioallantoic membranes of chicken embryos. Although the hemagglutinin of this virus could not be distinguished from that of vaccinia, the lesions produced on the chorioallantoic membrane of chicken embryos by the 2 viruses differed. Complement-fixation tests indicate an antigen difference between the new agent and vaccinia virus. Results to date suggest that this agent is a new and distinct member of the variola-vaccinia group of viruses, but the exact relationship to the other members of the group has not been established.

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The Use of Trichloroethylene and Halothane Anesthesia in the Restraint of Laboratory Primates

ROY KINARD, D.V.M., and CHARLES W. McPHERSON, D.V.M.

Bethesda, Maryland

THE SEARCH for an anesthetic agent that could be used safely and simply to restrain potentially dangerous laboratory primates has led to successful trials of two such agents—trichloroethylene* and halothane** (2-bromo-2-chloro-1,1,1-trifluoroethane). Both are volatile liquid halogenated hydrocarbons possessing the following characteristics: (1) Their vapors in air or oxygen are noninflammable, nonirritating, and of pleasant odor; (2) both induce anesthesia rapidly in relatively low concentration; and (3) both induce anesthesia with an acceptable margin of safety.

Trichloroethylene boils at 87.6 C., its specific gravity is 1.47, and its molecular weight is 131.4. The anesthetic grade of trichloroethylene is tinted with "waxoline" blue and contains 0.01 per cent thymol.

Injuries to man due to exposure to industrial grades of trichloroethylene have been reported, but the toxicity was probably due to impurities.[2] Rats, rabbits, and dogs exposed to subinduction concentrations (0.05 to 0.2%) of chemically pure trichloroethylene vapor for periods up to six months showed no adverse functional or anatomical effects.[4, 9]

Trichloroethylene has been used as an analgesic and anesthetic agent in thousands of human patients.[5, 8] Induction and recovery are rapid and postoperative discomforts are rare. Tachypnea often occurs during anesthesia and is considered a sign of overdose.[5] Cardiac arrhythmias are sometimes observed but are usually transient and not dangerous.

Trichloroethylene has been used by a single-dose method for rapid tooth extractions and tonsillectomies in groups of English children.[1] Induction, by means of a mask and rebreathing apparatus, required about two minutes. Surgery was completed without further administration after induction.

Halothane is a clear, colorless liquid with a boiling point of 50.2 C., specific gravity of 1.86, and molecular weight of 197.4.

Raventos[6] reported on trials of halothane in several species of laboratory animals. The therapeutic index of halothane for mice was greater than that of ether or chloroform but not as great as that of trichloroethylene. Dogs breathed halothane in induction concentration (3 to 4%) in air for 20 to 60 minutes before respiratory arrest occurred. Both induction and recovery were rapid and remarkably free of excitement, salivation, and vomiting. The use of halothane caused no functional disturbance except increased sensitivity of the heart to adrenalin during anesthesia.

Halothane has been used widely as the sole or principal anesthetic agent in human patients.[7] Induction and recovery are rapid and smooth, and unpleasant complications are rare. There is usually decreased pulse rate, blood pressure, and respiratory depth, with increased respiratory rate, under halothane anesthesia. Cardiac arrhythmia sometimes occurs but is not considered serious in most instances.

Krantz *et al.*,[3] in toxicity studies, found no evidence of injury to rats following chronic exposure to halothane vapor. They reported severe respiratory depression and sometimes irreversible respiratory arrest in dogs and monkeys under halothane anesthesia.

METHODS

Trichloroethylene and halothane were administered in a transparent chamber (fig. 1). The chamber frame and floor were made of wood; the sides and top, of wire-reinforced glass; and the vertically sliding doors, at each end, of Plexiglas. The chamber was 18 inches wide, 23 inches high, and 24 inches long (inside measurements). Its volume (inside) was 5.75 cubic feet.

The monkeys and chimpanzees were readily coaxed into the chamber directly from their cages. The anesthetic, in liquid state, was sprayed onto the inside walls of the chamber with a blunt hypo-

From the Animal Hospital Section, Division of Research Services, National Institutes of Health, Bethesda, Md.

* Trimar, product of Ohio Chemical and Surgical Equipment Co., Madison, Wis.

** Fluothane, product of Imperial Chemical Industries, Ltd., Manchester, England.

dermic needle inserted through a ⅛-inch hole in one of the doors. Evaporation occurred immediately in most instances and never required more than one minute.

Dosages of trichloroethylene and halothane are stated in percentage vapor in air by volume. To determine the volume of vapor produced by evaporation of a given volume of liquid, the following formula was used:

$$\text{liters vapor} = \frac{22.4 \text{ ml. of liquid} \times \text{sp. g. liquid}}{\text{molecular weight}} \times \frac{\text{temperature (A)}}{273}$$

Calculations based on this formula revealed that 1 per cent vapor in 1 cubic foot of air (at 78 F. or 299 A., the usual temperature in the chamber) is produced by the following volumes of liquid: trichloroethylene 1.0 ml., halothane 1.2 ml. To produce 1 per cent vapor in the chamber (5.75 cu. ft.) required 5.8 ml. of trichloroethylene or 7.0 ml. of halothane.

Preliminary trials showed that the lowest vapor concentration which consistently caused rapid induction in rhesus monkeys was 2.7 per cent for both trichloroethylene and halothane. Ten monkeys (7 rhesus, 2 cynomolgus, and 1 African green) were anesthetized with 2.7 per cent trichloroethylene vapor, then removed from the chamber, and allowed to recover. Time required for induction and recovery, the respiratory rate and character, and the pulse rate and character were recorded. One week later, this procedure was repeated using the same monkeys and 2.7 per cent halothane vapor.

Both drugs were used on a trial basis to restrain large monkeys and chimpanzees for tuberculin tests. As a result of these trials, vapor concentrations were increased to 3.2 per cent trichloroethylene or halothane for monkeys and to 4.2 per cent trichloroethylene for chimpanzees. Finally, the method described was adopted for routine restraint of monkeys and chimpanzees weighing over 10 lb.

RESULTS

Trichloroethylene.—Induction with trichloroethylene vapor, 2.7 to 3.0 per cent, in monkeys was usually smooth until the animal lay down. There was occasional salivation or slight muscular tremors or stiffness but never signs of irritation or discomfort before the animal lost consciousness. Following loss of consciousness, tachypnea and rigid partial flexion of the limbs invariably occurred. In the 10 monkeys administered trichloroethylene vapor, 2.7 per cent, the time required to lose consciousness varied from one to two minutes, and the respiratory rate immediately after loss of consciousness varied from 50 to 132, with the mean of 87.6 per minute. The tachypnea always continued as surgical anesthesia was reached.

As soon as the animal was removed from the chamber, the respiratory rate began to decrease. Immediately after removal, the pulse rate was too high to be counted accurately. At this time, relaxation was usually fair, sometimes good, and anesthesia was satisfactory for injections and brief surgical procedures.

The signs of impending awakening consisted of dilation of the pupils, nystagmus,

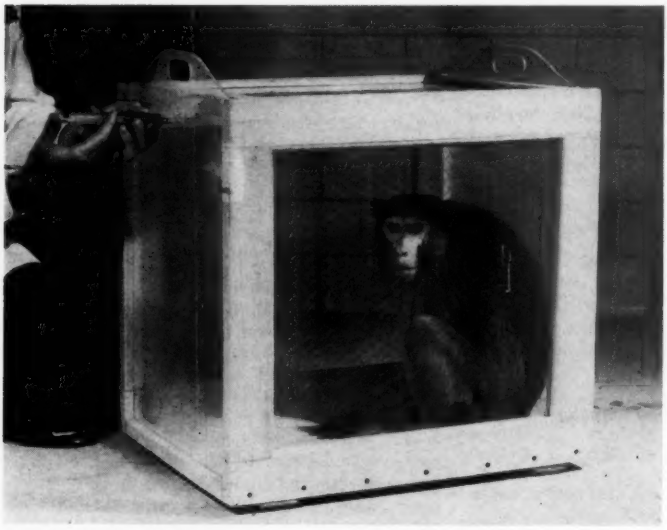

Fig. 1—The anesthetic is sprayed onto the inside walls of the chamber.

and rigid flexion of the limbs. Recovery was gradual, with no danger of sudden attack by the animal. On being replaced in its cage, the monkey would regain equilibrium quietly and without excitement. It would sit upright while gradually regaining normal control of all faculties.

The times required for induction and recovery of 20 monkeys and 17 chimpanzees administered various concentrations of trichloroethylene vapor are shown (table 1). The only advantage demonstrated for the use of 3.2 per cent vapor, rather than 2.7 per cent, in monkeys is the longer minimum time required for them to become potentially dangerous when the greater concentration is used.

The reactions of chimpanzees to trichloroethylene vapor were the same as those of monkeys except that the chimpanzees recovered much more rapidly. It can be seen (table 1) that the time required for chimpanzees to become potentially dangerous increased as vapor concentration was increased. However, time for complete recovery was not increased correspondingly.

Countless monkeys and chimpanzees have been restrained by this method, some of them several times, using trichloroethylene vapor, 3.2 to 4.2 per cent. In spite of the disadvantages, particularly the very high respiratory and pulse rates produced, no known death, injury, or illness has resulted from its use.

Whenever large numbers of monkeys were to be handled, 2 or 3 were placed in the chamber together. Under these circumstances, the dosage of trichloroethylene was not changed, and no difference in the result was observed.

Halothane.—It was observed that induction with halothane vapor, 2.7 per cent and 3.2 per cent, was smoother and more rapid than with ether, chloroform, or trichloroethylene. There was no evidence of discomfort, excitement, salivation, or stiffness during induction. The subject often yawned before loss of consciousness. Frequently, the loss of equilibrium was so rapid that the animal collapsed suddenly. Some monkeys would arise and collapse again before losing consciousness. The respiratory rate was increased moderately during induction but was not nearly as rapid as induction with trichloroethylene. In the 10 monkeys administered halothane vapor, 2.7 per cent, the time required for loss of consciousness varied from one to three minutes, and the respiratory rate immediately after loss of consciousness varied from 32.0 to 56.0, with the mean of 45.6 per minute. The respiratory rate slowed as the state of surgical anesthesia was reached. Occasionally, respiratory arrest occurred. In every case, respiratory arrest was reversed by removing the monkey from the chamber and applying manual artificial respiration for one minute or less. Pulse rates, counted immediately after removal of the animals from the chamber, were 80 to 100 per minute. Transient cardiac arrhythmia, consisting of skipped beats and lasting about one minute, was detected in 1 cynomolgus monkey when it was removed from the chamber.

Recovery from halothane anesthesia was also exceptionally smooth and rapid. The monkeys sometimes awakened suddenly, without warning. This could have constituted a danger if the handlers had not been alert.

TABLE 1—Time, in Minutes, Required for Induction and Recovery in Monkeys and Chimpanzees Administered Trichloroethylene and Halothane Vapors in a Chamber

Anesthetic	Per cent	No. animals	Time to surgical anesthesia and removal from chamber**		Time to become potentially dangerous†		Time to recover†	
			Range	Mean	Range	Mean	Range	Mean
Trichloroethylene	2.7	10 monkeys*	3–5	4.0	2–16	9.0	5–30	16.2
Trichloroethylene	3.2	10 rhesus monkeys	3–6	4.1	5–15	9.1	13–30	20.3
Trichloroethylene	3.2	5 chimpanzees	3–5	4.0	1–2	1.2	6–10	7.4
Trichloroethylene	3.7	7 chimpanzees	3–6	3.7	1–4	2.3	2–6	4.0
Trichloroethylene	4.2	5 chimpanzees	2–5	3.4	2–4	3.0	4–10	5.8
Halothane	2.7	10 monkeys*	2–7	4.7	2–7	3.0	5–14	8.7
Halothane	3.2	15 rhesus monkeys	2–4	3.1	1–5	3.3	4–15	6.4

* Seven rhesus, 2 cynomolgus, 1 African green monkey; various weights up to 40 lb. ** Measured from the time of the introduction of the anesthetic into the chamber. † Measured from the time of the removal of the animal from the chamber.

XUM

The times required for induction and recovery of 25 monkeys administered 2.7 per cent or 3.2 per cent halothane vapor are shown (table 1).

Three monkeys suffered respiratory arrest during induction with halothane. Each was resuscitated, and no known harm resulted. To investigate this problem further, each of 5 monkeys was exposed to halothane vapor by means of an ether cone until respiratory arrest occurred. In each case, cessation of exposure to the vapor and manual artificial respiration resulted in recovery without apparent injury. Seven additional monkeys were allowed to remain in the chamber for five minutes following routine induction with halothane, and none developed respiratory arrest. In contrast to the findings of Krantz *et al.*,[3] irreversible respiratory arrest did not occur in any of the animals tested in this study. The 200 administrations of halothane to monkeys in this series have produced no known unfavorable result except reversible respiratory arrest.

CONCLUSION

Trichloroethylene has been adopted for routine use, but halothane has not. The former drug is less expensive and allows a longer recovery period without the possibility of sudden awakening. The only advantage of halothane over trichlorethylene is its smoother action.

Since simplicity and economy were primary objectives, certain refinements, such as (1) addition of ventilating or spraying apparatus to the chamber, (2) addition of oxygen to the anesthetic-air mixture, and (3) use of the drugs in combination, were not tested in this series.

Although the procedure described here is neither hazardous nor uncomfortable to the subjects, it should be used only on those animals whose attitude toward human interference is either hostile or unknown. For animals of docile nature, or those to be handled often, suitable training is preferable to frequent anesthetic restraint.

SUMMARY

Trichloroethylene vapor, 3.2 to 4.2 per cent, and halothane vapor, 3.2 per cent, in air, were administered to monkeys and chimpanzees in a transparent chamber. This procedure proved an effective and safe means of temporary restraint of the animals and has been adopted for routine use for that purpose in the primate colony in which it was tested.

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Occurrence of the Nematode, *Anatrichosoma cutaneum*, in the Nasal Mucosae of *Macaca mulatta* Monkeys

ANTON M. ALLEN, D.V.M.

Bethesda, Maryland

In 1922, Swift *et al.*[3] described nematode infection in the skin of the extremities of a number of *Macaca mulatta* monkeys and proposed the name, *Trichosoma cutaneum*, for the nematode. In 1954, Smith and Chitwood [2] described a new trichuroid nematode found in the nasal mucosa of *Macaca philippinensis* monkeys. These workers assigned the nematode to the family Trichuridae and proposed a new subfamily, Anatrichosominae. They named the genus Anatrichosoma and the nematode *Anatrichosoma cynamolgi*. More recently, 1958, Chitwood and Smith [1] transferred *Trichosoma cutaneum* to the genus Anatrichosoma, the correct designation for this species now being *Anatrichosoma cutaneum* (Swift, Boots, and Miller, 1922) Chitwood and Smith, 1958. In their 1958 paper, they concurred with Skrjabin *et al.*, 1954, in referring the subfamily Anatrichosominae to the family Trichosomoididae, instead of the family Trichuridae.

Infection with *A. cutaneum* in the nasal mucosa of *M. mulatta* evidently has not been described in the literature. This report pertains to the occurrence of nasal infections with the nematode and the lesions that are produced.

Methods

The monkeys, members of the quarantine colony of the National Institutes of Health, were *Macaca mulatta* averaging 2 to 3 years of age. They were held as pairs in double-deck steel dog cages and housed in air-conditioned rooms containing 50 cages. They were fed commercially prepared laboratory biscuits with vitamin C. Food and water were supplied in containers attached to the outer surfaces of the cage doors.

The noses were excised from 17 randomly selected monkeys at the time of routine necropsy and were fixed in neutral 10 per cent formalin. Sections were cut transversely through the nasal septum and both nasal passages, including the alar cartilages, and stained with hematoxylin and eosin. Nematode infection of the nasal mucosa was determined by the presence of the parasite in the tissues on microscopic examination. Nematode specimens were later obtained for identification by dissection of the fixed tissues. Parasite identification was performed and reported by M. B. Chitwood.[1]

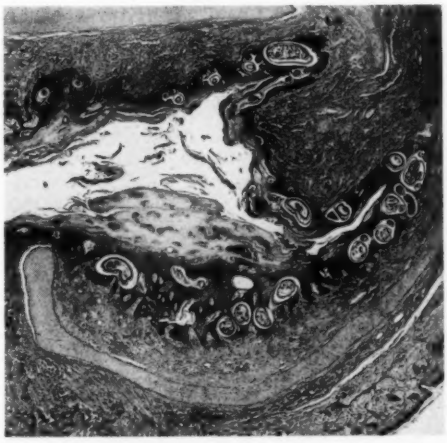

Fig. 1—Section from the nasal passage of a monkey, including the septum and alar cartilage. Severe infection by Anatrichosoma cutaneum is evident. H & E stain; x 17.

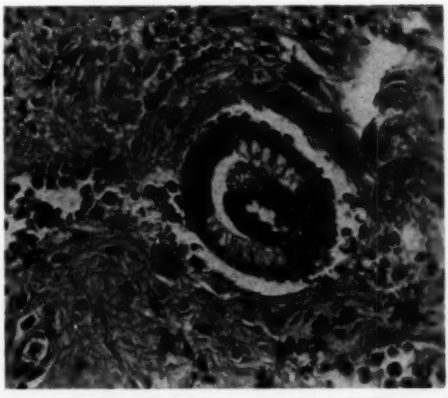

Fig. 2—Section through a nematode in the lumen of a necrotic blood vessel in the lamina propria of the nasal mucosa of a monkey. H & E stain; x 310.

From the Comparative Pathology Section, Laboratory Aids Branch, Division of Research Services, National Institute of Health, Bethesda, Md.

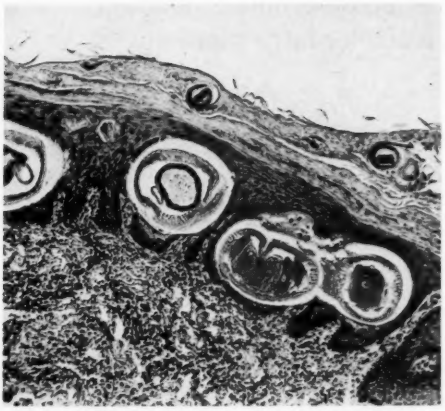

Fig. 3—Section through the nasal mucosa of a monkey demonstrating the epithelial enclosure of the nematodes, moderate hyperplasia, and nematode ova in the overlying parakeratotic plaque. H & E stain; x 80.

PATHOLOGY

Necropsy of the 17 monkeys revealed no significant gross lesions in the nasal passages. Occasionally, a firm, gray-white material, indistinguishable from dry mucous secretions, was adhered to the mucosal surface.

Examination of histologic sections of the nasal structures revealed moderate to severe infections by *A. cutaneum* in 6 of the 17 monkeys. The nematodes were observed in the stratified squamous epithelial layer of the nasal mucosa (fig. 1). In 3 of the monkeys, nematodes were also located within vessels of the lamina propria. The inhabited vessels appeared to be veins or venous sinuses; however, focal necrosis and distension of the vessels hampered accurate identification (fig. 2). Occasionally, nematodes were located in the connective tissue of the lamina propria with no apparent vascular enclosure.

Diffuse hyperplasia and parakeratosis were the most prominent changes observed in the mucosal epithelium (fig. 1, 3). The severity of the hyperplastic change was roughly proportional to the severity of the infection, and the thickness of the involved epithelium was increased from two to ten times normal. The nematodes were located near the base of the epithelial layer within large intra-epithelial spaces. The spaces appeared to be artifacts produced by shrinkage of the nematodes during fixation. The walls of the spaces were formed by flattened epithelial cells, each space conforming to the shape of the enclosed nematode (fig. 3). Hyaline remnants of nematodes were observed occasionally within the spaces. Frequently two nematode bodies occupied one space, but it was not determined whether they represented two nematodes or different portions of one nematode. Often the two bodies differed considerably in diameter, one measuring 60 to 72 μ and another measuring 156 to 202 μ. The larger bodies, containing numerous ova, were obviously portions of female parasites (fig. 4). The

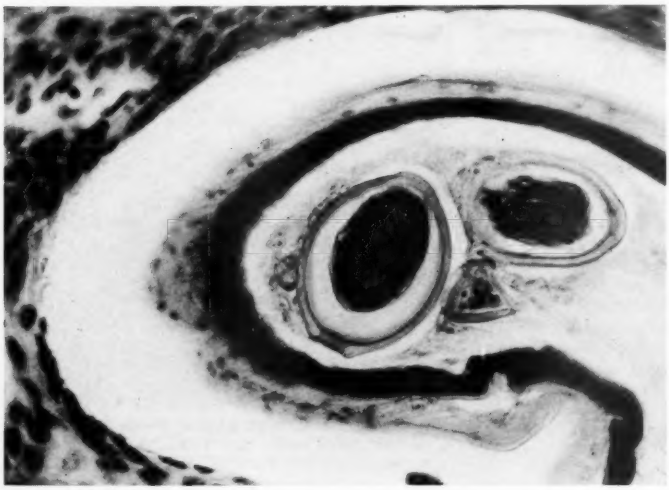

Fig. 4—Section through an intra-epithelial nematode containing typical opercular ova. H & E stain; x 392

biopercular ova were observed also in the plaques of nucleated keratin on the surface of the epithelium (fig. 3, 6). They were embryonated, elliptical in shape, and were 40 to 48 by 60 to 67 μ in diameter. The segments of nematodes in the lamina propria were 58 to 72 μ in diameter and contained no visible ova.

The inflammatory reactions in the lamina propria varied in intensity. The sections from 1 monkey showed no reaction. In the remaining 5, lymphocytes, plasma cells, and eosinophils were moderately increased in number, especially at the junction of the lamina propria with the epithelial layer. The most severe inflammatory reactions were observed in the sections from the 3 monkeys having intravascular parasites. Neutrophils were increased in number and many thin-walled vessels were necrotic. An unusual perivascular granulomatous reaction was observed around many vessels. The predominant cells were spindle-shaped and resembled reticular cells of lymphoid tissue, differing from fibroblasts in having little cytoplasm and small, intensely basophilic nuclei (fig. 5). Mononuclear and polymorphonuclear leukocytes were scattered among these cells. Fibroplasia and capillary proliferation were minor features of the perivascular lesions.

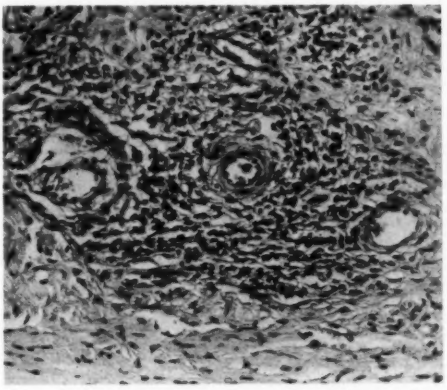

Fig. 5—Section through the nasal lamina propria of a monkey. The numerous perivascular cells resemble lymphoid reticular cells. H & E stain; x 160.

Discussion

Infection of the nasal mucosa of 6 *M. mulatta* by *A. cutaneum* resulted in minimal to moderate tissue reactions, but, at the time of necropsy, had caused no gross lesions considered characteristic of the condition. The histopathologic studies indicated that the female parasites are located only in the squamous epithelial layer of the mucosa, and that the ova are deposited in the epithelium, finally being shed from the mucosal surface in plaques of exfoliating, keratinized cells. Parasites presumed to be the male form of the species, or immature forms of either sex, inhabited thin-walled vessels of the lamina propria.

The *A. cutaneum* infections described by Swift *et al.*[3] occurred in the skin of *M. mulatta*, primarily involving the extremities. There is no indication that the nasal passages of these monkeys were examined. Generally speaking, the lesions were similar to the nasal lesions, since large female nematodes were located in the squamous epithelium of the skin, and small nematodes of undetermined sex were located subcutaneously. However, some characteristics of the skin lesions differed from the nasal lesions. For example, the skin lesions evidently lacked significant hyperplasia and parakeratosis. Furthermore, Swift *et al.* described a subcutaneous foreign-body reaction, characterized by the presence of multinucleated giant cells and marked fibroplasia, which ultimately resulted in encapsulation and destruction of the subcutaneous nematodes. If the subcutaneous nematodes were located within vessels, this feature of the lesion was apparently obscured by the granulomatous tissue reac-

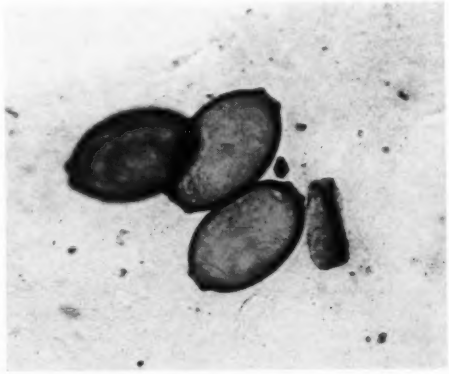

Fig. 6—Photomicrograph of a fragment of keratinized epithelium scraped from the nasal mucosa of a monkey, showing the dense, unstained, reddish-brown ova of Anatrichosoma cutaneum. H & E stain; x 285.

tion. Finally, Swift *et al.* described "epithelial blisters" which ruptured and discharged ova from the skin surface.

Although the skin lesions of *A. cutaneum* were evidently striking in appearance, their occurrence has not been reported since Swift's report in 1922. Considering this fact, in addition to the severity of the skin lesions in comparison to the nasal lesions, it seems likely that skin may be an aberrant location of the nematode. Considerably more might be learned about the natural location of the parasite by a thorough search of the skin of monkeys known to have nasal infections. Nasal infection can be recognized antemortem by demonstrating ova in scrapings of the anterior nasal mucosa (fig. 6).

The lesions caused by *A. cynamolgi* infections in *M. philippinensis* have not been described. The author has had occasion to examine the infected nasal mucosa of only 1 monkey of this species. The lesions were similar to those of *A. cutaneum* in *M. mulatta*. However, the intravascular nematodes were observed in subcutaneous vessels of the skin overlying the alar cartilages. The inhabited vessels appeared to be thin-walled veins.

SUMMARY

Seventeen monkeys of the species *Macaca mulatta* were randomly selected at the time of routine necropsy, and were examined for the presence of nasal parasites. Six of the monkeys were found to be infected by *Anatrichosoma cutaneum*. The nematodes were located in the squamous epithelium and the lamina propria of the nasal mucosa.

While some of the nematodes of the lamina propria evidently were free in the connective tissue, most of them were located within vessels. Chronic inflammatory reaction of minimal to moderate severity was present in the nasal laminae propriae of 5 of the monkeys.

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Immunohematologic Studies of the Thoroughbred Horse

MANUEL A. GILMAN, V.M.D.; ALFRED SCHWARZ, M.D., F.C.A.P.; HARRY WALLERSTEIN, M.D.

New York, New York

THE IMPORTANCE of blood-grouping and blood-matching studies in veterinary medicine increases with the knowledge that refinements in techniques can help reduce the dangers of blood transfusions; weed out some undesirable breeding characteristics; aid in problems of sterility; extend our knowledge of the relationship between blood groups; guide in the determination of disputed parentage; and elucidate the etiology of hemolytic anemia in the newborn, a condition which occurs spontaneously in horses and man.

More than 1,100 Thoroughbred horses were used in studies on blood-grouping and blood-matching. Such a large number of test animals seemed necessary because of conflicting findings reported in the literature.

Balakrishnan and Yeravdekar [2,3] classified blood of horses in four groups, comparable to the A, B, and O groups in man. These findings were confirmed by Bruner and Doll [4] who used stock serums supplied by Balakrishnan and Yeravdekar and performed experiments on 376 horses, some of which were Thoroughbreds. Adams,[1] on the other hand, defined two blood groups on the basis of naturally occurring antibodies which he claims are found in the absence of the corresponding antigens not related to the human A and B antigens. Matsumoto and Watanabe [5] defined nine antigens; Podliachouk [6] claimed to have found ten erythrocytic antigens in horses, 162 of which were Thoroughbreds. Schermer and Kaempffer [7] reported the occurrence of six antigens which they labeled A, B, C, D, E, and F, with the corresponding antibody occurring in some but not all of the animals lacking the antigen. Our work confirms most of the findings of the latter even though we used our own testing serums.

From the Research Laboratories of the Jewish Memorial Hospital, New York 40, N.Y.

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The authors thank Horace Wilson, M.T., for technical assistance; also Mr. Marshall Cassidy, executive secretary of the Jockey Club, and Dr. Alexander Kaye for their help and encouragement; and the many horse owners for granting permission to use their horses for blood grouping, isoimmunization, and transfusion studies.

MATERIALS AND METHODS

More than 1,100 Thoroughbred horses of both sexes were used. These ranged in age from yearlings to 25 years. They were representative of the breed and included horses in training, stallions, and brood mares, as well as some yearlings.

Blood Grouping.—We used the direct test tube technique which matches the serum of the recipient against the blood cells of the donor. The serum was obtained as follows: Blood from horses previously found to contain not more than one type of isoantibody was collected and placed in sterile vacuum containers. Anticoagulants were not used. The blood was allowed to clot, the serum separated and 0.1 cc. of 1 per cent solution of sodium azide (as preservative) was added to each 10.0 cc. of serum. These, the testing serums, were so labeled and kept under refrigeration.

The absorption technique was used to eliminate multiple antibodies in the serum. Erythrocytes were washed four or more times with normal saline solution until the supernatant fluid was clear. Equal volumes of erythrocytes and serum were mixed in a tube measuring 10 by 70 mm. The mixture was incubated at 30 C. and the erythrocytes resuspended every 15 minutes for one hour. The mixture was centrifuged and the absorbed serum removed into a clean tube and tested for complete absorption with a fresh set of erythrocytes of the same horse. If no agglutination occurred, the serum was ready to be used; if positive, the procedure was repeated until agglutination was absent.

Blood to be typed was taken with a regulation bleeding needle from the jugular vein directly into a sterile 20-cc. glass tube. It was allowed to clot, the serum was removed, and the cells were resuspended in normal saline solution. The cells were washed and centrifuged four times with normal saline; this resulted in clear washings. A 5 per cent suspension of cells was made. In a 10- by 70-mm. tube, 3 drops of testing serum added to 3 drops of 5 per cent suspension of cells were mixed and incubated at 37 C. for 30 minutes, centrifuged at 1,500 r.p.m. for one minute, and examined under the microscope for evidence of agglutination. All tests were made in duplicate. If any discrepancies occurred, the blood in question was retyped in triplicate.

Blood cross-matching tests were performed with undiluted blood, bovine albumin, enzyme-treated

erythrocytes, and blood cells suspended in normal saline. After the first 250 horses were tested, the normal saline suspension method was resorted to exclusively, since results with this method were identical with those obtained by the more intricate procedures.

In the normal saline-suspension method, two Kahn tubes were used for each cross-matching test. Tube 1 was labeled "recipient's cells and donor's serum"; tube 2, "recipient's serum and donor's cells." To each of these tubes were added 1 drop of blood cells (diluted with 1 drop of physiologic saline) and 1 drop of serum, as labeled (*i.e.*, recipient's blood cells with donor's serum into tube 1 and donor's blood cells with recipient's serum into tube 2). The tubes were held at room temperature for 30 minutes, centrifuged at low speed for one minute, tapped gently, and contents examined macroscopically for hemagglutination. Also, the contents of each tube were placed on a glass slide and examined thoroughly under low magnification for evidence of clumping or lack of clumping.

RESULTS

Six different, naturally occurring testing serums, each with one type of antibody, were obtained with the procedures outlined above. These serums were labeled anti- a, b, c, d, e, and f. All six were used to type each horse's red blood cells. It was found that six different types of antigens are present in the Thoroughbred horse's red blood cells. These antigens were labeled A, B, C, D, E, and F. The erythrocytes of individual horses may contain any one of these antigens, any combination of them, or none of them. Since possession of antigens by the red blood cells is the factor which determines the blood group, it is possible to test for 56 different blood types in the Thoroughbred horse. Perhaps as more studies are made, other antigens will be identified, additional testing serums will be made for ascertaining the presence or absence of these antigens, and more blood types will be discovered in the Thoroughbred horse. If a horse's red blood cells were agglutinated by anti- a and f testing serums, this horse was labeled type AF. If a horse's red blood cells were agglutinated by all six testing serums, then this horse was called type ABCDEF, and so on. If none of the testing serums agglutinated a horse's red blood cells, then it was classified as type X.

The incidence of 56 blood groups has not been definitely established. Twenty-four different blood types were obtained out of a possible 56 types in 102 Thoroughbred horses selected at random and tested for blood groupings. Type X, the serum of which contains no isoantibodies and the blood cells of which contain no antigens, was found in 18 of the 102 random blood specimens (*see* table 1).

TABLE 1—Incidence of Blood Types in 102 Thoroughbred Horses—24 Out of a Possible 56

Serums						Blood types	No. of horses
a	b	c	d	e	f		
○	○	○	○	○	○	X	18
●	●	●	●	○	●	ABCDF	9
●	○	●	●	○	●	ACDF	8
●	○	●	○	○	●	ACF	8
●	○	●	●	●	●	ACDEF	7
○	○	●	○	○	●	CF	6
●	○	●	●	○	○	ACD	6
○	○	○	○	○	●	F	5
●	○	●	○	○	○	AC	5
●	○	●	○	●	●	ACEF	5
●	●	●	●	●	●	ABCDEF	4
●	●	●	○	●	●	ABCEF	3
●	○	○	○	○	●	AF	3
●	●	●	○	○	●	ABCF	2
○	●	●	●	○	●	BCDF	2
○	○	●	○	○	○	C	2
○	○	○	○	●	●	EF	2
○	○	●	○	●	○	CE	1
●	○	○	○	●	●	AEF	1
○	○	○	○	●	○	E	1
●	○	○	●	○	●	ADF	1
○	○	●	●	○	●	CDF	1
●	●	●	●	○	○	ABCD	1
●	○	○	○	○	○	A	1

● = Agglutination of red blood cells. ○ = No agglutination of red blood cells.

Hereditary Transmission of the Blood Groups.—Isoantibodies in the horse are natural antibodies and exist from birth. In this respect, they parallel the major blood group antibodies in man (anti-A and anti-B). Of 341 Thoroughbred horses tested (random sampling) isoantibodies were found in only 21 (11 brood mares and 10 horses in training—7 males and 3 females). Of the 11 brood mares whose blood contained isoantibodies, 8 produced foals with hemolytic disease. Preliminary studies indicate that the blood of the foal cannot have isoantigens that are not found in the blood of the sire or dam or both (*see* table 2). If the sire and dam are type X, then the foal must be type X. Most of these antigens are heterozygous and this accounts for the fact that often there is loss of one or more antigens in the blood of the foal that was present in the blood of the sire or dam.

DISCUSSION

Tests on 1,100 Thoroughbred horses indicate that the horse may fall into any one of 56 well-defined blood groups, based on agglutination reactions. Further studies may reveal more blood groups. All agglutination reactions depend upon two factors —the substance causing the reaction (isoantibody found in the serum portion of the blood) and the substance acted upon (isoantigen found in red blood cells). In man, if a particular antigen is present, then the agglutinating antibody is always absent. This is also true in the horse. In man, if a particular antigen or antigens are absent, the opposing antibody or antibodies are always present with some rare exceptions. In the Thoroughbred horse, however, this does not hold true—the antibodies may be present or absent.

Successful multiple whole blood transfusions in the Thoroughbred horse can be carried out when group-specific blood is used. In other words, a donor of type F can be used safely with a recipient of type F, EF, AEF, etc., as long as the serum of neither horse contains antibodies. In such cases, the donor's blood antigen is not foreign to the recipient, even though of a different blood type.

The universal donor in the Thoroughbred horse is type X; the serum contains no isoantibodies and the red blood cells contain no antigens. This type of blood can apparently be given safely to any horse.

Preliminary studies on the hereditary transmission of blood groups in the Thoroughbred horse indicate that antigenic properties of the red blood cells are inherited similarly to those in man. If the sire and dam are type X, then the foal must be type X. The foal cannot have isoantigens that are not found in the blood of the sire or the dam or both. Isoantibodies are natural antibodies and exist from birth. In this respect, they are similar to the major blood group antibodies in man (anti-A and anti-B). They are apparently not the result of specific isoimmunization and thereby differ from the rhesus monkey (Rh) and similar antibodies.

The serum of the great majority of Thoroughbred horses does not contain demonstrable isoantibodies. Of 341 horses tested (random sampling), the serum of only 21 contained isoantibodies. Foals of 8 of 11 brood mares *with* isoantibodies had hemolytic anemia.

The solution of many problems dependent on blood-grouping and blood-matching studies awaits the availability of large amounts of testing serums.

TABLE 2—Heredity of Blood Groups in Thoroughbred Horses

Sire No.	Dam No.	Blood type		
		Sire	Dam	Foal
2064	2058	ACD	CDF	X
2064	2060	ACD	X	X
2064	2061	ACD	X	A
2064	2059	ACD	AC	ACD
2064	2057	ACD	X	AC
2064	2065	ACD	ACF	X
2064	2070	ACD	CF	AC
2064	2068	ACD	ACDF	AC
2064	2071	ACD	CF	X
2064	2066	ACD	AC	X
2063	2067	ABCD	ACDF	AF
2063	2069	ABCD	CF	X
2087	2092	X	X	X
2087	2094	X	ACD	A
2087	2089	X	X	X
2087	2197	X	AC	A
2087	2196	X	ACF	X
2087	2093	X	X	AF*

* Questionable parentage.

SUMMARY AND CONCLUSIONS

1) Blood specimens of 1,100 Thoroughbred horses were studied and six basic testing serums were found.

2) With these six serums, the presence or absence of six different isoantigens in the red blood cells of the horse were ascertained.

3) Theoretically, Thoroughbred horse's blood can be any one of 56 types, although thus far only 24 different types of blood have been identified in 102 horses tested by random sampling.

4) The great majority of horses do not contain demonstrable isoantibodies in their serums.

5) From the limited number of families of horses studied, it seems that the antigenic properties of red blood cells are inherited similarly to man.

6) Multiple whole blood transfusions based on selection of group specific blood may be safely given.

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Field Studies Comparing Piperazine-Carbon Disulfide Complex with Carbon Disulfide for Parasite Control in the Horse

J. H. DRUDGE, D.V.M., Sc.D.; S. E. LELAND, Jr., Ph.D.; Z. N. WYANT, B.S., A.M.; G. W. ELAM; L. B. HUTZLER, M. S.

Lexington, Kentucky

THE PRESENT OBSERVATIONS extend a previous field evaluation [4] of the piperazine-carbon disulfide complex* as an anthelmintic in the horse. A direct comparison of this compound with carbon disulfide was made with special attention to the control of ascarid infection. Until recently, carbon disulfide has been used in central Kentucky for ascarid infection, although there is evidence [12,13] of its lack of efficacy and undesirable toxic reactions often attend its use. In contrast, the piperazines have been generally characterized as relatively free of toxic side effects and consistently effective for the removal of ascarides from the horse.[1-11]

MATERIALS AND METHODS

The 1957 crops of foals on three central Kentucky Thoroughbred farms were used in this study. Observations were started on the animals when they were sucklings, continued through the weanling interval, and finally terminated in the summer of the yearling period. The parasitic infections were naturally acquired. The animals on each farm were allowed to graze together until they became yearlings, when the males were separated from the females. The animals were allotted to the treatment groups on the basis of age and sex. The attending veterinarians estimated body weights at each treatment and administered the drugs by stomach tube. The animal groupings, dosage rates, and other special treatment procedures used on the three farms were as follows:

Farm D.—Twenty-four animals were placed in three groups and were treated with (1) a suspension* of piperazine disulfide complex at the rate of 0.5 oz./100 lb. of body weight, (2) the suspension at 0.25 oz./100 lb., or (3) carbon disulfide at the approximate rate of 1 dr. (4.0 cc.) of the liquid per month of age, with a maximum dose of 6 dr. The animals were confined to their stalls during the night preceding treatment and were not fed the usual morning grain ration on the day of treatment. In addition, suckling foals given the carbon disulfide were muzzled for three hours before and two hours after treatment. The farm D animals were not on low-level phenothiazine medication for strongyle control, except during the suckling interval when they had access to the drug that was fed (2.0 Gm./day) to the mares. Inasmuch as separate feed boxes were provided for the foals, they ordinarily consumed little of the mares' grain ration containing the phenothiazine.

Farm L.—Twenty foals, placed in two groups, were given either the piperazine-carbon disulfide complex at the rate of 0.5 oz. of the suspension per 100 lb. of body weight, or carbon disulfide at the approximate rate of 2.4 to 4.5 cc. per 100 lb. of body weight. The grain ration was withheld only on the morning of the day of treatment. This farm purported to use low-level phenothiazine treatment for strongyle control. However, the application was generally poor because the drug was given sporadically.

Farm M.—Three groups were formed from 22 foals, and the group treatments were: (1) piperazine-carbon disulfide complex at the rate of 0.5 oz. of the suspension per 100 lb. of body weight, (2) the complex at the rate of 0.25 oz. of the suspension per 100 lb., or (3) piperazine citrate at the rate of 40 mg. piperazine base per pound of body weight. The last group was subdivided into two groups during the yearling interval and treated with piperazine dihydrochloride at the rates of 20 and 40 mg. of piperazine base per pound, respectively. The grain ration was withheld only on the morning of treatment. This farm not only used the low-level phenothiazine system for strongyle control during the period of these observations, but it had also rigidly applied the system during the preceding 12-year period.

The comparative efficacies of the various treatments were judged from a series of worm eggs per gram (e.p.g.) counts on fecal samples which were routinely made at two-week intervals. Ascarid, strongyle, and Strongyloides eggs were differentiated in the counting method which was the same as that described earlier.[4]

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* Parvex, manufactured by The Upjohn Company, Kalamazoo, Mich.

* A suspension of 250 mg. of piperazine-disulfide complex per cubic centimeter was used in all instances.

TABLE 1—Worm Egg Count Data on Suckling Foals

No. of animals	Preparation	Treatment: Dose Rate	Treatment: Dose Total	Eggs per gram of feces (group average): Pretreatment A	Pretreatment S	Post-treatment (week) 2 A	2 S	4 A	4 S	6 A	6 S	8 A	8 S	10 A	10 S
FARM D															
8	Piperazine complex	0.5 oz./100 lb.*	52–64 cc.	100	390	0	65	0	40	0	105	1	510	65	255
8	Piperazine complex	0.25 oz./100 lb.**	24–30 cc.	590	210	30	110	5	60	55	305	50	405	35	520
8	Carbon disulfide	1.0 dr./month	4–5 dr.	485	635	270	500	410	415	6†	180	0	140	0	140
FARM L-1															
10	Piperazine complex	0.5 oz./100 lb.	45–75 cc.	240	10	0	0	10	15	1	235	85	120	285‡	130‡
10	Carbon disulfide	2.4 cc./100 lb.	2–3 dr.	680	15	730	125	1,725	205	390‖	210	685	310	30‡	260‡
FARM L-2															
8	Piperazine complex	0.5 oz./100 lb.	60–90 cc.	55	780	0	5	0	5	0	10	10	35	125	165
7	Carbon disulfide	3.5 cc./100 lb.	4–4.5 dr.	965	355	70	60	50	175	100	55	35	95	60	170
FARM M															
8	Piperazine complex	0.5 oz./100 lb.	60–90 cc.	5	110	0	10	0	10	0	30	0	30	0	30
8	Piperazine complex	0.25 oz./100 lb.	30–40 cc.	15	170	0	40	0	30	0	70	0	110	0	115
6	Piperazine citrate	40.0 mg.§/lb.	16–18 Gm.§	0	35	0	50	0	5	0	10	0	10	0	10

Farm M animals were on a low-level phenothiazine treatment.

A = ascarid; S = strongyle.

* Delivered approximately 20 mg. of piperazine base per pound and 1.75 cc. of carbon disulfide per 100 lb. of body weight. ** Delivered approximately 10 mg. of piperazine base per pound and 0.88 cc. of carbon disulfide per 100 lb. of body weight. † Treated again during the four- to six-week interval. ‡ Average of 3 animals. ‖ Four animals treated again during the four- to six-week interval. § Piperazine base.

TABLE 2—Worm Egg Count Data on Weanling Foals

No. of animals	Preparation	Treatment: Dose Rate	Treatment: Dose Total	Eggs per gram of feces (group average): Pretreatment A	Pretreatment S	Post-treatment (week) 2 A	2 S	4 A	4 S	6 A	6 S	8 A	8 S	10 A	10 S
FARM D															
16	Piperazine complex	0.5 oz./100 lb.*	52–90 cc.	80	415	0	15	2	10	2	155	15	425	125	670
16	Piperazine complex	0.25 oz./100 lb.**	24–45 cc.	35	355	7	30	0	35	0	190	20	275	165	400
15	Carbon disulfide	.....	5 dr.	50	235	0	270	0	245	0	360	40	400	200	720
FARM L															
12	Piperazine complex	0.5 oz./100 lb.	60–90 cc.	40	75	0	5	0	5	0	140	1	180	25†	345†
9	Carbon disulfide	4.0 cc./100 lb.	5–6 dr.	100	190	0	295	0	141	0	170	5	150	95†	720†
FARM M															
7	Piperazine complex	0.5 oz./100 lb.	98–105 cc.	75	55	0	1	0	3	0	4	0	10	4	40
8	Piperazine complex	0.25 oz./100 lb.	45–55 cc.	10	180	0	10	0	10	0	25	0	25	1	45
6	Piperazine citrate	40.0 mg.‡/lb.	24–48 Gm.‡	5	50	0	0	0	2	0	2	0	2	10	15

Farm M animals were on a low-level phenothiazine treatment.

A = ascarid; S = strongyle.

* Delivered approximately 20 mg. of piperazine base per pound of body weight and 1.75 cc. of carbon disulfide per 100 lb. ** Delivered approximately 10 mg. of piperazine base per pound of body weight and 0.88 cc. of carbon disulfide per 100 lb. † 12 weeks post-treatment. ‡ Piperazine base.

Detailed observations on the individual animals during the early phases of this study have been published elsewhere.[5]

RESULTS

Sucklings.—The worm egg counts of the suckling foals are summarized (table 1). On farm D, first treatment was given when the foals were 3 to 4 months old. Only one treatment with the complex was administered, while two treatments with carbon disulfide were given one month apart. The treatment dates with the complex were July 5 and August 15 for the early and late foals, respectively. The treatment dates with carbon disulfide were July 5 and August 15 for the early foals and August 15 and September 12 for the late foals. The egg count data for the early and late foal subgroups were combined, along with data following the second treatment of the carbon disulfide group as a continuation beyond the fourth post-treatment week (table 1).

The first medication of the farm L sucklings was given on June 11, when 13 animals 8 weeks of age and older were treated. One of the 6 suckling foals in the carbon disulfide group that did not respond was given a second dose of carbon disulfide one month later on July 8, when the 7 younger foals, then 2 months old, were first treated. The egg count data for these two treatment dates were combined (L-1, table 1). August 25 was the second general treatment for the farm L sucklings that were first treated in June (L-2, table 1). On this date, a second dose of carbon disulfide was also administered to the 4 foals that were given their first carbon treatment in July, but the data on these animals are included in L-1 as a continuation beyond the fourth post-treatment week.

Farm M sucklings were treated only once (Aug. 20) and treatment was intentionally delayed until ascarid eggs were present in the feces of several animals. The low-grade level of infection on this farm apparently minimized the exposure to infection, and thus made the first appearance of the infection relatively late in comparison with farms D and L.

On all three farms the piperazine-carbon disulfide complex at 0.5 oz./100 lb. came close to completely eliminating ascarid eggs from the feces. There was no significant return until the tenth week. Half this dosage level (0.25 oz./100 lb.) was also completely effective on farm M, and reduced the average post-treatment ascarid egg count in the farm D weanlings about 95 per cent.

In contrast, on both farms D and L, the initial dose of carbon disuulfide lowered the average ascarid egg count less than 50 per cent, and a return to pretreatment levels was observed by the fourth post-treatment week. A second treatment with carbon disulfide before the sixth week was quite effective on farm D, but on farm L a transient reduction of only 75 per cent in ascarid e.p.g. was observed.

Strongyle egg counts on the sucklings were (1) markedly lowered by the 0.5 oz./100 lb. dosage of the complex on all three farms, (2) reduced to a lesser degree by the 0.25 oz./100 lb. dosage of the complex on farms D and M, and (3) essentially unchanged by the carbon disulfide treatment on farms D and L. They were further reduced by piperazine treatment from the relatively low levels maintained by low-level phenothiazine medication on farm M.

Weanlings.—Observations on the animals during the weanling interval are also summarized (table 2).

Only one treatment was used on two of the farms (Nov. 5 on farm L and Dec. 31 on farm M). On farm D the foals were treated twice, the first at weaning time (Sept. 12 for the early foals and Oct. 10 for the late foals) and the second on December 5, when all animals were dosed. Egg count data for these treatments on farm D are combined and presented as one group for each drug and dose rate (table 2).

The piperazine-carbon disulfide complex at both dosage levels, the carbon disulfide, and the piperazine citrate were uniformly highly effective in reducing post-treatment ascarid egg counts. Mostly negative counts were recorded until the eighth week, when a low-grade reappearance became evident on farms D and L in both the complex and carbon disulfide treated animals. Relatively high counts were observed on the tenth week on farm D.

Strongyle egg counts were effectively reduced by the complex at both dosage levels, and by the piperazine citrate, while the carbon disulfide had no apparent effect. Significant increases of the strongyle egg counts in the piperazine-treated animals were recorded on the sixth post-treatment

zine base per pound of body weight and 0.88 cc. of carbon disulfide per 100 lb. † 12 weeks post-treatment. ‡ Piperazine base.

TABLE 3—Worm Egg Count Data on Yearlings

No. of animals	Preparation	Treatment: Dose: Rate	Treatment: Dose: Total	Eggs per gram of feces (group average): Pretreatment: A	Pretreatment: S	Post-treatment (week): 2: A	2: S	4: A	4: S	6: A	6: S	8: A	8: S	10: A	10: S
FARM D-1															
23	Piperazine complex	0.5 oz./100 lb.*	90 cc.	365	855	0	10	0	5	0	65	0	200	50	85
FARM D-2															
5	Piperazine complex	0.5 oz./100 lb.*	90–115 cc.	135	180	0	5	0	5	0	185	0	355	40	295
7	Piperazine complex	0.25 oz./100 lb.**	52–60 cc.	35	125	0	35	0	25	0	55	0	195	0	305
6	Carbon disulfide	.....	6 dr.	160	265	0	165	0	620	0	330	0	660	0	650
FARM L															
11	Piperazine complex	0.5 oz./100 lb.*	90 cc.	55	620	0	295	..	.....	..	.....	..	.....	4	790
11	Carbon disulfide	4.5 cc./100 lb.	6–7 dr.	115	890	0	800	..	.....	..	.....	..	.....	2	765
FARM M															
6	Piperazine complex	0.5 oz./100 lb.*	115–120 cc.	60	55	0	0	0	0	10	0	3	0	10	10
8	Piperazine complex	0.25 oz./100 lb.**	52–68 cc.	110	185	15	5	20	10	25	25	5	20	40	35
3	Piperazine dihydrochloride	40.0 mg.†/lb.	26–32 Gm.†	70	35	3	3	55	10	10	0	0	3	0	10
3	Piperazine dihydrochloride	20.0 mg.†/lb.	14–16 Gm.†	65	85	7	15	0	0	3	7	0	3	3	20

Farm M animals were on a low-level phenothiazine treatment.
A = ascarid; S = strongyle.
* Delivered approximately 20 mg. of piperazine base per pound of body weight and 1.75 cc. of carbon disulfide per 100 lb. ** Delivered approximately 10 mg. of piperazine base per pound of body weight and 0.88 cc of carbon disulfide per lb. † Piperazine base.

week on farms D and L. Relatively low counts persisted throughout the period of observation on farm M, where the low-level phenothiazine was also used.

Yearlings.—The egg count observations during the yearling period are also summarized (table 3).

Farm L and farm M animals were treated once (March 3 or April 2), those on farm D were treated twice (March 5 and May 25). For the March 5 treatment on farm D, each animal was given 3 oz. of the complex and the egg count data were recorded separately (D-1, table 3). The regular treatment groupings were resumed for the May 25 dosing on farm D, and the egg count data are given (D-2, table 3).

Both dosage levels of the complex and the carbon disulfide were uniformly effective in reducing the ascarid egg counts to negative or low levels two weeks following treatment; characteristically, no evidence of a return was seen until the tenth week. The use of piperazine dihydrochloride on farm M was followed by less effective reductions of ascarid egg counts.

Strongyle egg counts were consistently reduced following treatment with the piperazines, while no change was effected by the carbon disulfide. The reductions associated with the piperazine treatment ranged from 60 per cent by the complex at 0.5 oz./100 lb. on farm L to 100 per cent by the same treatment on farm M. Reductions were maintained through the fourth week, after which marked increases were generally observed except on farm M, where the phenothiazine treatment resulted in low average counts throughout the period of observations.

DISCUSSION

The superior efficacy of the piperazine-carbon disulfide complex over carbon disulfide for ascarid control was most evident in this study in the suckling age group. One treatment with piperazine markedly reduced and held the ascarid egg counts to negative or very low levels through an eight-week post-treatment period. Two treatments with carbon disulfide during the same interval produced only transient and partial reductions of the average ascarid e.p.g. Some animals showed no change in e.p.g. after treatment, which indicated the failure to remove adult ascarides; more commonly, post-treatment reductions were only transient, which indicated a lack of

efficacy against the immature ascarides. These shortcomings of carbon disulfide have been reported previously.[12,13]

In the older animals (weanlings and yearlings), the efficacy of carbon disulfide against the ascarid compared favorably with the piperazine-carbon disulfide complex. Both compounds were consistently near 100 per cent effective in eliminating ascarid eggs from the feces with no reappearance until a lapse of eight weeks. This indicated that both preparations were active against the immature as well as the mature worms in the older animals.

The contrast in the activity of carbon disulfide between the sucklings and the older animals is of special interest. The generally lower dosages and the minimal fasting procedure may have accounted for these activity shortcomings in the farm L sucklings. However, the more favorable combination of higher dosages and more rigid fasting, including pre- and post-treatment muzzling, did not improve the results of carbon disulfide therapy on farm D. Elimination of these factors on this basis suggests that there in an additional factor common to both farms. Milk in the diet is the most apparent difference between the sucklings and the older animals and could be reasonably held accountable for the activity differences; the implication was that the more rigid fasting on farm D still did not provide for sufficient emptying of the stomach of the "blocking" agent.

The use of the piperazine-carbon disulfide complex at the hitherto untested rate of 0.25 oz. of the suspension per 100 lb. of body weight resulted in a consistently high order of activity against the ascarid. This action from total doses of the suspension as low as 24 cc. in the sucklings was particularly impressive. At this rate, piperazine base was delivered at the approximate rate of 10 mg. per pound of body weight. Thus, the complex compares favorably with polymethylenepiperazine, which has been evaluated by critical tests[6] at this low dosage rate. On the basis of published reports, both of these compounds appear to be more active at lower doses than other piperazines. However, testing at dosages below 40 mg. of base per pound is limited and additional investigations are in progress to study the relationship of activity to dosage with other piperazine salts.

Limited experience with piperazine dihydrochloride indicated that it was somewhat less effective for removal of ascarides than the piperazine-carbon disulfide complex. This was observed in animals given dihydrochloride at the rate of 40 mg. of base per pound, which is twice the amount of base delivered by 0.5 oz. of the suspension of the complex per 100 lb. of body weight. Treatment of 12 sucklings and weanlings with piperazine citrate at the relatively high rate of 40 mg. of base per pound resulted in 100 per cent removal of ascarides.

In this study, intervals between treatments with piperazine were at least ten weeks; this gave sufficient time for the developmental cycle of the ascarid in the animals. Thus, the series of post-treatment fecal examinations for reappearance of ascarid eggs served as a measure of drug efficacy against both mature and immature worms and also determined the actual existence of the infection in the animals. The regularity with which the ascarid eggs reappeared or the counts increased after the post-treatment interval of eight weeks agrees with previous observations[4] and establishes the existence of the constant exposure to the infection throughout the period of observations on all three farms. The absence of ascarid eggs through the eight-week post-treatment periods supports the previous observation[4] that spacing piperazine treatments at two-month intervals will effectively control ascarid infection under the conditions in which it occurs in young horses in this area.

In comparison with the carbon disulfide, which was not active on strongyles, piperazine provided the additional activity against these forms which was manifested in the marked reductions of strongyle egg counts after treatment. These reductions were characteristically maintained through a four-week post-treatment interval, after which increases occurred. Of particular interest was the effectiveness of the relatively low dosage rate (0.25 oz./100 lb.) of the piperazine-carbon disulfide complex. The activity was probably limited to the small strongyles, because previous observations[4] indicated that even 0.5 oz./100 lb. was too low for removal of the large strongyle, *Strongylus vulgaris*. The lack of action against the large strongyles should not discount the value of the low dosage levels of piperazine in sucklings and weanlings, because the large strongyles generally have not reached the intestinal tracts

of animals less than 6 months old and thus are not subject to the removal action of the drug. The magnitude of the initial large strongyle infections in these young animals reflects the general level of control achieved by the measures applied to the mares and is not affected by the direct treatment of the sucklings and weanlings.

The effectiveness of the low-level system of phenothiazine treatment for strongyle control was also quite evident in this study. A much lower order of strongyle e.p.g. was associated with the regular administration of the drug on farm M than on the other two farms where the system was used only sporadically or not at all. These low counts on farm M also indicated that the prolonged use of the system during the preceding 12-year period had not resulted in a loss of effectiveness due to the development of a drug-resistance by the strongyles. Although the pretreatment counts in farm M animals were relatively low, marked reductions regularly followed piperazine treatment. However, the usual reappearance of strongyle eggs that is seen after the fourth week following piperazine therapy was not observed in the farm M animals.

SUMMARY

The 1957 foals on three central Kentucky farms were followed during the first year of development by a series of biweekly fecal examinations for worm eggs to compare the effectiveness of piperazine-carbon disulfide complex with carbon disulfide for parasite control, with special attention to ascarid infection.

In suckling foals, the piperazine-carbon disulfide complex was definitely more effective than carbon disulfide for ascarid control. The observed shortcomings of carbon disulfide were (1) incomplete action in removing mature worms, (2) no apparent action at all in an occasional animal, and (3) incomplete removal of immature ascarides. Although the complex at 0.25 oz./100 lb. of body weight (10 mg. piperazine base/lb.) was much more active than the carbon disulfide, this dosage rate is below that necessary (0.5 oz./100 lb.) for the most effective control obtainable from this preparation.

In older animals (weanlings and yearlings), carbon disulfide and the piperazine were equally effective for the removal of both mature and immature ascarides.

Limited trials with piperazine citrate at 40 mg. of piperazine base per pound indicated complete removal of mature and immature ascarides from sucklings and weanlings. In contrast, piperazine dihydrochloride at 20 and 40 mg. of base per pound showed a marked but incomplete action on ascarid infection in yearlings.

The various dosage levels of all three piperazine compounds were consistently effective in reducing strongyle egg counts during a four-week post-treatment period, after which increases occurred. The antistrongyle activity, particularly of the low dose rates, was of interest but was probably limited to the small strongyles.

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Infectious Meningo-Encephalitis in Cattle, Caused by a Haemophilus-like Organism

PETER C. KENNEDY, D.V.M., Ph.D.; ERNST L. BIBERSTEIN, D.V.M., Ph.D.;
J. A. HOWARTH, D.V.M., Ph.D.; LILLIAN M. FRAZIER;
D. L. DUNGWORTH, B.V.Sc., M.R.C.V.S.

Davis, California

IN OCTOBER, 1958, a feedlot operator in the delta area of the central valley of California began sustaining severe losses of a type that was new to him and us. This report discusses the clinical and pathologic details of this disease and its experimental reproduction by a previously unreported bacterium.

CLINICAL FEATURES

The first deaths which occurred in this feedlot, presumably caused by this disease, were not indicative of a new problem. The cattle were found either dead or *in extremis*. The losses were sporadic, only 1 animal in a pen usually being involved, but after two weeks the tempo of the disease increased and 6 animals died on the first of November. All showed signs of brain disease. The neurologic signs were extremely variable. Most commonly noticed were stupor, opisthotonus, ataxia, weakness, and paralysis. At this time, the owner began a careful twice-a-day inspection, riding through each pen and making each animal move. By this inspection system, the disease could be recognized in a preparetic form. Animals in the early stages of the disease were stiff, reluctant to move, and febrile, with temperatures up to 107 F. At this stage the animal could be successfully treated, but those untreated until they developed focal neurologic signs made much less satisfactory recoveries. Those that were first recognized when showing signs of severe generalized central nervous system disorder invariably died.

Although the feeding and management practices followed at this feedlot were marginal, during the preceding years the death loss due to all causes was less than 0.1 per cent. During the five months following the recognition of the disease, approximately 75 animals (2.5%) died of what the owner believed was this entity. It was nearly impossible to determine the morbidity of the disease in the feedlot. The owner was convinced that antibiotic treatment was successful if begun early (the authors agree). He treated at least 800 animals which he thought were in the prodromal stage of the disease, and they responded to the treatment. It is likely that at least half (400) were correctly diagnosed.

The 3,000 cattle in the feedlot were allotted to 15 pens according to their stage of fattening. The pattern of the epizootic was unusual in that the cattle in no single pen appeared to be the focus of infection. During the five months of the epizootic, the sickness continued to be randomly distributed in the yards.

Necropsy Findings.—Eight of the animals which died were necropsied and the lesions which we came to accept as characteristic of the disease were observed. A small, gram-negative organism, which will be discussed, was recovered from 3 of these animals and from 1 other.

The characteristic lesions were confined to the brain. Multiple or single hemorrhagic foci could be seen in any part of the brain (fig. 1). The general pattern of these lesions and their association with thrombosed vessels indicate their infarct nature. Their diameter ranged from 1 to 4 cm. The color of these lesions also varied from the bright red of recent hemorrhage to older areas of brownish discoloration. Meningeal changes consisted of a slight opalescence or clouding of cerebrospinal fluid surrounding the older superficial brain lesions. Lesions not involving the central nervous system were less conspicuous and less specific. They included excess fluid in the pericardial sac and joint capsules and hemorrhages in the serosa of the heart. Small hemorrhages in the muscles and kidneys were the same type of vascular lesions seen in the brain but were much less striking.

Histopathology.—The most characteristic lesion of this disease was a bacterial vascu-

From the University of California, School of Veterinary Medicine, Davis.

of animals less than 6 months old and thus are not subject to the removal action of the drug. The magnitude of the initial large strongyle infections in these young animals reflects the general level of control achieved by the measures applied to the mares and is not affected by the direct treatment of the sucklings and weanlings.

The effectiveness of the low-level system of phenothiazine treatment for strongyle control was also quite evident in this study. A much lower order of strongyle e.p.g. was associated with the regular administration of the drug on farm M than on the other two farms where the system was used only sporadically or not at all. These low counts on farm M also indicated that the prolonged use of the system during the preceding 12-year period had not resulted in a loss of effectiveness due to the development of a drug-resistance by the strongyles. Although the pretreatment counts in farm M animals were relatively low, marked reductions regularly followed piperazine treatment. However, the usual reappearance of strongyle eggs that is seen after the fourth week following piperazine therapy was not observed in the farm M animals.

SUMMARY

The 1957 foals on three central Kentucky farms were followed during the first year of development by a series of biweekly fecal examinations for worm eggs to compare the effectiveness of piperazine-carbon disulfide complex with carbon disulfide for parasite control, with special attention to ascarid infection.

In suckling foals, the piperazine-carbon disulfide complex was definitely more effective than carbon disulfide for ascarid control. The observed shortcomings of carbon disulfide were (1) incomplete action in removing mature worms, (2) no apparent action at all in an occasional animal, and (3) incomplete removal of immature ascarides. Although the complex at 0.25 oz./100 lb. of body weight (10 mg. piperazine base/lb.) was much more active than the carbon disulfide, this dosage rate is below that necessary (0.5 oz./100 lb.) for the most effective control obtainable from this preparation.

In older animals (weanlings and yearlings), carbon disulfide and the piperazine were equally effective for the removal of both mature and immature ascarides.

Limited trials with piperazine citrate at 40 mg. of piperazine base per pound indicated complete removal of mature and immature ascarides from sucklings and weanlings. In contrast, piperazine dihydrochloride at 20 and 40 mg. of base per pound showed a marked but incomplete action on ascarid infection in yearlings.

The various dosage levels of all three piperazine compounds were consistently effective in reducing strongyle egg counts during a four-week post-treatment period, after which increases occurred. The antistrongyle activity, particularly of the low dose rates, was of interest but was probably limited to the small strongyles.

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Infectious Meningo-Encephalitis in Cattle, Caused by a Haemophilus-like Organism

PETER C. KENNEDY, D.V.M., Ph.D.; ERNST L. BIBERSTEIN, D.V.M., Ph.D.;
J. A. HOWARTH, D.V.M., Ph.D.; LILLIAN M. FRAZIER;
D. L. DUNGWORTH, B.V.Sc., M.R.C.V.S.

Davis, California

IN OCTOBER, 1958, a feedlot operator in the delta area of the central valley of California began sustaining severe losses of a type that was new to him and us. This report discusses the clinical and pathologic details of this disease and its experimental reproduction by a previously unreported bacterium.

CLINICAL FEATURES

The first deaths which occurred in this feedlot, presumably caused by this disease, were not indicative of a new problem. The cattle were found either dead or *in extremis*. The losses were sporadic, only 1 animal in a pen usually being involved, but after two weeks the tempo of the disease increased and 6 animals died on the first of November. All showed signs of brain disease. The neurologic signs were extremely variable. Most commonly noticed were stupor, opisthotonus, ataxia, weakness, and paralysis. At this time, the owner began a careful twice-a-day inspection, riding through each pen and making each animal move. By this inspection system, the disease could be recognized in a preparetic form. Animals in the early stages of the disease were stiff, reluctant to move, and febrile, with temperatures up to 107 F. At this stage the animal could be successfully treated, but those untreated until they developed focal neurologic signs made much less satisfactory recoveries. Those that were first recognized when showing signs of severe generalized central nervous system disorder invariably died.

Although the feeding and management practices followed at this feedlot were marginal, during the preceding years the death loss due to all causes was less than 0.1 per cent. During the five months following the recognition of the disease, approximately 75 animals (2.5%) died of what the owner believed was this entity. It was nearly impossible to determine the morbidity of the disease in the feedlot. The owner was convinced that antibiotic treatment was successful if begun early (the authors agree). He treated at least 800 animals which he thought were in the prodromal stage of the disease, and they responded to the treatment. It is likely that at least half (400) were correctly diagnosed.

The 3,000 cattle in the feedlot were allotted to 15 pens according to their stage of fattening. The pattern of the epizootic was unusual in that the cattle in no single pen appeared to be the focus of infection. During the five months of the epizootic, the sickness continued to be randomly distributed in the yards.

Necropsy Findings.—Eight of the animals which died were necropsied and the lesions which we came to accept as characteristic of the disease were observed. A small, gram-negative organism, which will be discussed, was recovered from 3 of these animals and from 1 other.

The characteristic lesions were confined to the brain. Multiple or single hemorrhagic foci could be seen in any part of the brain (fig. 1). The general pattern of these lesions and their association with thrombosed vessels indicate their infarct nature. Their diameter ranged from 1 to 4 cm. The color of these lesions also varied from the bright red of recent hemorrhage to older areas of brownish discoloration. Meningeal changes consisted of a slight opalescence or clouding of cerebrospinal fluid surrounding the older superficial brain lesions. Lesions not involving the central nervous system were less conspicuous and less specific. They included excess fluid in the pericardial sac and joint capsules and hemorrhages in the serosa of the heart. Small hemorrhages in the muscles and kidneys were the same type of vascular lesions seen in the brain but were much less striking.

Histopathology.—The most characteristic lesion of this disease was a bacterial vascu-

From the University of California, School of Veterinary Medicine, Davis.

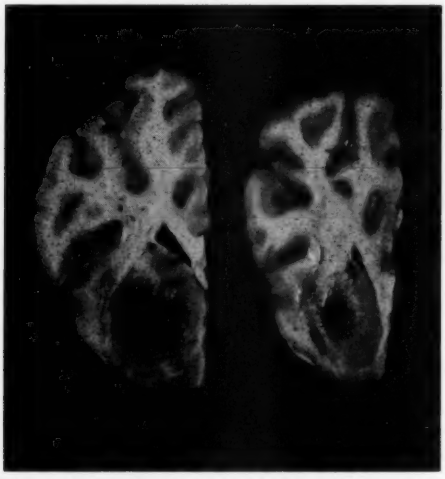

Fig. 1—Hemorrhagic foci of inflammation and infarction in the brains of 2 cattle with field cases. The random distribution of the lesions was typical.

litis which led to thrombosis and subsequent septic areas of infarction involving the brain. What were interpreted as various stages of the development of this lesion were frequently seen in a single section. Capillaries and venules were occluded by masses of bacteria (fig. 2, 3). The inflammatory reaction associated with this bacterial colonization was variable; occasionally no change in the vessel wall was seen, but more frequently an acute vasculitis resulted (fig. 4). It appeared that this reaction rapidly involved the surrounding brain tissue, leading in turn to thrombosis of large vessels (fig. 5). Although many of these secondary thrombi were suppurative, few contained the large colonies of bacteria seen in the capillary and venule thrombi, and in all cases vasculitis was present. The inflammatory reaction, wherever present, was acute and the cellular response was almost entirely neutrophilic. The effects of the infarction and inflammation in many areas overlapped, but the lesions were sharply demarcated from the surrounding tissue.

Although the lesion was best and most frequently seen in the brain, other organs were involved. The essential features of the lesion remained the same, but its consequences depended in part on the vascular supply of the organ. It was clear from both the gross and microscopic examination that this lesion had a predilection for the brain, but it was impossible to state the extent of this predilection except in general terms.

ETIOLOGY

Cultures of brain lesions, kidneys, spleen, liver, lungs, muscle lesions, synovial fluid, and (under certain conditions) blood yielded a bacterium which we have neither

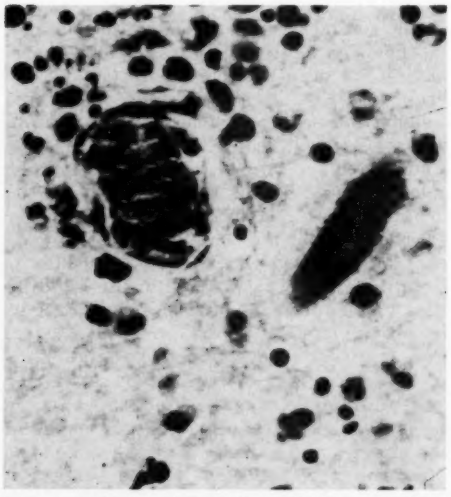

Fig. 2—Colonies of bacteria occluding a cerebral venule and capillary. Minimal inflammatory changes were present at this stage. Barbeito-Lopez; x 460.

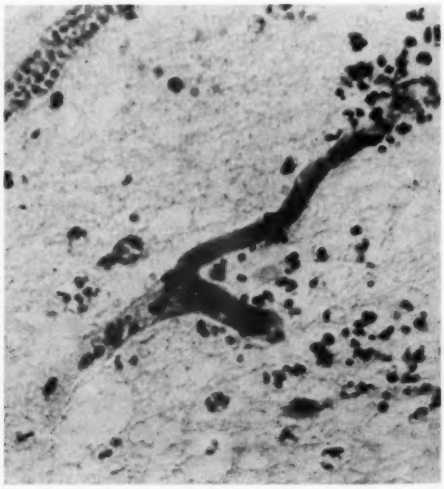

Fig. 3—Bacterial thrombus in a small cerebral venule in which a vasculitis is developing. Barbeito-Lopez; x 230.

encountered previously nor found described in the literature. It will, therefore, be described in some detail.

Morphology.—The organism was a small coccobacillus ranging from almost coccoid forms, 0.3 to 0.4 μ in diameter by 0.8 μ in length, to bacilli 0.3 to 0.5 μ in diameter by 1.5 to 4.0 μ in length. Short filaments, chains of cocci, and some bizarre forms were seen. The morphologic diversity was not so apparent after several transfers on artificial mediums. The organism stained well with the usual dyes and was gram-negative. It was nonmotile and nonsporulating. No capsules could be demonstrated from cultures or from the peritoneal fluid from mice that had been inoculated intraperitoneally with broth cultures.

Culture Characteristics.—Isolation attempts were most frequently successful when tissue blocks or relatively large volumes of fluid were placed in a liquid medium, such as brain-heart infusion broth, and incubated overnight at 37 C. under 5 to 10 per cent carbon dioxide. Subcultures were made the following morning by dropping several drops of the incubated broth onto the surface of beef blood agar plates. In positive cases, transparent, pinpoint-sized, nonhemolytic colonies appeared after another overnight incubation period under raised carbon dioxide tension. Blood isolations were readily obtained by collecting blood from febrile animals directly into flasks of brain-heart infusion broth, 50 ml. of blood being added to 100 ml. of medium. Under these conditions, a loose clot gradually formed and abundant growth was detectable in the supernatant fluid within 18 to 24 hours. The organisms often appeared aggregated into clumps and sometimes strongly suggested intracellular growth *in vitro* (fig. 6).

Colonies on blood agar were raised, circular, smooth, and entire. On longer incubation, the colonies showed a slightly granular appearance with a papillate center and flattened periphery. Long sinuous parallel filaments with ovoid swellings, as well as polar beading and chains of regular bacilliform cells, were seen in some colonies stained by Klineberger-Giemsa agar block technique.[4] The colonies on beef blood agar ranged from 0.2 to 0.6 mm. in diameter and did not enlarge appreciably under prolonged incubation.

With fresh isolates, no growth was obtained on infusion or trypticase soy mediums, serum enriched broth or agar, or any supplemented mediums, including hemoglobin agar, the ingredients of which had been exposed to standard autoclaving procedures. Nor could the organism be propagated under atmospheric or anaerobic conditions. Satellitism could not be demonstrated on blood or chocolate agar using several strains of staphylococci. On 10 per

Fig. 4—A well-developed vasculitis with neutrophils infiltrating through the damaged wall. H & E; x 115.

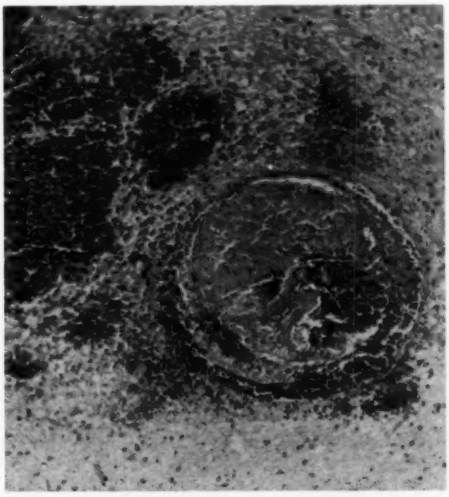

Fig. 5—Thrombosis of a larger cerebral vessel associated with an area of severe inflammation. H & E; x 115.

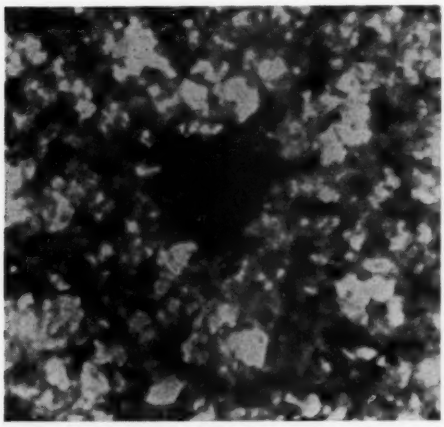

Fig. 6—Smear from a twenty-four-hour blood culture in brain-heart infusion broth. Gram stain; approx. x 2,000.

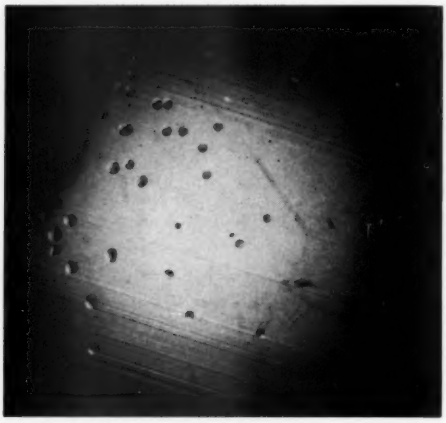

Fig. 7—Satellitism shown by colonies of the cattle encephalitis organism near a heavy streak of Staphylococcus aureus.

cent horse serum agar, however, good growth could be obtained in the vicinity of staphylococcal colonies (fig. 7).

After continuous culture on blood agar for two months, the organism could also be propagated on a number of mediums that previously had failed to support growth. These included serum broth and agar, hemoglobin agar (the constituents of which had been autoclaved for 30 minutes or more) and, eventually, infusion broth, although growth in this medium was never abundant. Attempted cultures in tryptose, peptone, and nutrient broth remained uniformly unsuccessful. One per cent yeast hydrolysate agar proved to be superior to blood mediums for propagation of the organism, and growth was equally abundant in cases where the yeast hydrolysate had been sterilized by Seitz filtration, as after autoclaving for 45 minutes.

A re-isolate of the organism from a brain frozen at –10 C. for two months grew well in tissue culture medium 199 (Difco)—a mixture of amino acids, purines, pyrimidines, vitamins, minerals, carbohydrates, and various defined trace substances, but devoid of coenzymes. Growth in this medium, however, was contingent upon maintenance of the pH above 6.0, which was accomplished by the addition of phosphate buffers.

Three months after isolation, the bacterium could be grown on yeast hydrolysate agar under atmospheric conditions. No growth had been obtained in a similar attempt four weeks previously.

Biochemical Characteristics.—Fermentative ability was tested in ordinary carbohydrate mediums enriched with one or two drops of fresh defibrinated blood. Slight acidity was produced in arabinose, xylose, glucose, rhamnose, maltose, mannose, and sorbitol. One of four isolates attacked sucrose and dulcitol as well. No change was observed in lactose, mannitol, raffinose, fructose, and inositol. Uninoculated control mediums were included in each series to detect any fermentative activity on the part of the added blood cells. There was a slight amount of indol produced. The test for nitrate reduction was very faintly positive. No hydrogen sulfide was formed and gelatin was not liquefied. There was some reduction apparent in litmus milk after seven days' incubation. MacConkey's bile salt agar did not support growth, even of well established culture.

Pathogenicity.—Mice were readily susceptible to intraperitoneal and intracerebral inoculation. Death occurred within 24 hours and the $l.d._{50}$ for intraperitoneal inoculation was estimated to be in the vicinity of 7,500,000 cells or 0.05 ml. of a 24-hour blood broth culture. The organisms were readily recovered from the peritoneal cavity and brain, respectively. Fresh isolates killed 2 of 3 guinea pigs within 18 to 24 hours following intraperitoneal inoculation of 0.5 ml. of broth culture. The organisms were present in great abundance in the peri-

toneal fluid where they occurred predominantly within neutrophils. Later passages of the organism failed to kill guinea pigs. One of 2 rabbits succumbed to intravenous injection of 2 ml. of blood broth culture of a recent isolate. Inoculation of 0.2 ml. of sodium chloride suspension from a 24-hour blood agar culture into the yolk sacs of 6-day chicken embryos consistently killed the embryo within 24 to 28 hours. A heavy growth of the organism was obtained on blood agar plates from the harvested yolk sac material.

Antibiotic Response in Vitro.—Bacto sensitivity disks of intermediate concentration were applied to blood agar plates that had been smeared with a 24-hour blood-trypticase soy broth. These plates were incubated under increased CO_2 tension. Chloramphenicol, dihydrostreptomycin, penicillin, polymyxin B, oxytetracycline, and erythromycin all inhibited the growth of the organism. Chlortetracycline, bacitracin, and neomycin showed no inhibition of growth.

The Experimental Disease.—In these experiments, two types of inoculums were used. The first calves in the series were inoculated with saline suspensions of the organisms which had been washed off blood agar slants. The organisms in these inoculums had been repeatedly transferred on blood agar. The erratic results of these experimental exposures led us to try to standardize the inoculum. For this purpose, blood samples from infected calves were collected directly into mediums and the resultant blood broth was used as inoculum. This technique was used because, in the course of these experiments, the subjective opinion was reached that cultivation on mediums resulted in a loss of pathogenicity.

Intravenous (i.v.) inoculation of the organism was lethal to 5 calves; these animals developed characteristic signs and lesions of the disease. The pattern of disease which developed in these 5 animals, while basically similar, showed variation. All animals became febrile, having temperatures over 105 F. Neither the height of the fever nor the speed with which it developed was well correlated with the length of survival. Survival varied from 24 to 96 hours and appeared related to the sensitiveness of sites of localization in the brain. All animals showed a stiffness and reluctance to move associated with a high fever. Two positive blood cultures were made at this stage. Frank neurologic signs were often associated with falling temperatures and failure to recover the organism from the blood.

Complete hemograms were irregularly done on these calves but, as judged by leukocyte counts done on 4 of these animals, the leukocyte response appeared to follow the pattern of other acute bacterial diseases. Leukopenia developed during the first 24 hours, followed by a leukocytosis if the animal survived. The leukocytosis was primarily caused by a neutrophilia with many immature forms present.

In the 1 calf inoculated intraperitoneally, the clinical signs were not typical of the field disease and the most obvious lesion at necropsy was a fibrinous peritonitis. The organisms were recovered from all organs cultured of the animals which died after experimental infection.

Experimental exposure in other animals was not uniformly successful in reproducing the disease. Two calves were resistant to infection at the first i.v. exposure. Two other calves were not made ill by nasal or ocular exposure and later did not react to i.v. inoculation. Two lambs failed to sicken after intravenous exposure. A small vaccination trial was inconclusive because the 4 calves which had been given the killed organisms did not become ill following i.v. challenge, and neither did the control calf.

Discussion

We are not aware of any similar disease having been described in cattle. Recently, however, Griner *et al.*[2] reported as a pathologic entity an infectious embolic meningo-encephalitis in cattle. Their pictures and descriptions were remarkably similar to those reported here. They concluded, however, that the condition they described resulted from a wide variety of primary disease conditions. A meningo-encephalitis described by Shand and Markson [5] appears to be primarily a meningitis with secondary involvement of the brain proper. Affected animals were exclusively calves, no more than 2 weeks old, and the bacterium recovered was identified as *Pasteurella septica.*

Our understanding of this disease is handicapped by the fact that our experience with the natural condition was limited to those extremely fulminating cases that were available to us. The fact that the experimental disease followed similar clinical and pathologic patterns makes it seem likely that the experiment duplicated at least some

of the features of the natural disease in this herd. We do not know whether this organism represents a pathogen which may be expected to cause disease in any feedlot or whether this herd was unique in some subtle and unrecognized way.

Evidence is presented to show that the clinical disease, even in its mildest form, represented the exception and that inapparent infection was the rule. At one point, about three months after the onset of the epizootic, the owner sent 83 steers, most which he believed had shown no recognizable illness, to a nearby slaughterhouse. Blood serum obtained from these animals was tested in a plate agglutination test against a strain isolated from the brain of a steer that had died earlier. The results of this test and tests on blood collected for unrelated purposes from cattle in two different herds are shown (table 1).

TABLE 1—Results of Plate Agglutination Tests Against a Strain of Coccobacillus from the Brain of an Affected Steer

	Affected herd	Control herd A	Control herd B
No. animals tested	83	50	60
No. positive in 5 min.	81	19	3
No. positive in 2 min.	75	14	1
No. positive in 1 min.	70	4	0
Av. No. sec. required for agglutination under 2 min.	34.8	82.5	115.0

No matter which of the three sets of results represents a valid sign of infection, the affected herd showed an incidence of reactors not less than 83 per cent, whereas the owner claims to have noticed illness in about 800 (27%) of the cattle; thus, roughly three times the number of clinically sick animals may be presumed to have been infected. The origin and method of spread of the offending organism is at present unknown. There are some rather suggestive parallels between the epidemiologic and pathogenic activity of this organism and that of epidemic meningitis due to *Neisseria meningitidis* in man; *i.e.*, the high incidence of infection compared to that of brain involvement, the septicemic phase preceding the more typical and severe sign of localization in the central nervous system, and the highly toxic effect of the organism on vascular tissue. The possibility that this bacterium may be an inhabitant of the upper respiratory tract and can give rise to epizootics manifested as septicemia and meningo-encephalitis, under special circumstances, has a great deal to recommend it as a working hypothesis.

The question regarding the place of this organism in a bacterial classification is difficult to decide at this time. When the isolates were fresh, all indications pointed to the genus Haemophilus as the proper taxonomic unit. Our inability to propagate these isolates on bloodless mediums and hemoglobin mediums that had undergone prolonged heating at autoclave temperature suggested that this organism required either one or both of the accessory factors necessary for members of the genus. When it was found that yeast hydrolysate alone was sufficient to support growth, the hemin-like, or X factor, appeared to be dispensible. When the growth-inducing principle in yeast hydrolysate proved to be stable for 45 minutes of exposure to 121 C., the V factor (as defined for Haemophilus [1]) also appeared unnecessary. It became obvious in the course of these tests that the organism had undergone changes in its metabolic requirements as shown also by its gradual adaptation to atmospheric environment. That some accessory factor is still needed is evident from continued failure of the organism to multiply in such mediums as tryptose broth and trypticase soy broth in the absence of some supplement, its striking response to yeast hydrolysate, and formation of satellite colonies around staphylococcal growth on otherwise inadequate mediums.

From the point of view of cellular morphology and certain culture traits, the present bacterium shows a great deal of resemblance to *Haemophilus agni,* isolated by Kennedy *et al.*[3] from lambs in a flock where an epizootic of a septicemic disease occurred. It differs in its absolute dependence on increased carbon dioxide tension in the case of fresh isolates, its pathogenicity pattern for experimental animals, and its eventual growth on bloodless and hemoglobin-free mediums. However, a close relationship between the two organisms is suggested by a series of complement-fixation tests in which *H. agni* reacted strongly with cattle serums containing antibodies to the encephalitis organism.

Apart from similarities between the two bacteria, the disease patterns associated with them show some striking parallels. Both infections tended to persist on the premises for several months, affecting indi-

vidual animals suddenly and disastrously. Experimentally, neither disease spread to contact animals and the natural diseases were reproduced only by intravenous inoculation. Intraperitoneal inoculation led in both instances to a fibrinous peritonitis. The basic lesion in both diseases is an acute bacterial thrombophlebitis.

SUMMARY

An epizootic of bacterial encephalitis in feedlot cattle is reported. The disease was experimentally reproduced by means of a previously undescribed organism.

Clinically, this disease was characterized by a brief prodromal stage of fever and stiffness followed by the rapid development of neurologic signs and death, if untreated.

Diagnostic gross lesions were confined to the brain, where single or multiple hemorrhagic areas of softening were present. Microscopically, these lesions showed evidence of a bacterial vasculitis which led to thrombosis and infarction.

The causative agent of this disease is a small, gram-negative coccobacillus which requires blood and an increased CO_2 tension for growth on primary isolation. The taxonomic aspects of this organism are discussed.

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Incidence of Parasites in Calves in Hawaii and the Treatment of *Cooperia punctata*, with Special Reference to the Efficacy of Ruelene

JOSEPH E. ALICATA, Ph.D.

Honolulu, Hawaii

IN HAWAII, beef and dairy calves often show signs of unthriftiness and fail to attain the desired rate of growth. Anorexia, diarrhea, and even deaths have occurred, especially in areas of high rainfall. During the past few years an investigation has been conducted to determine the extent to which gastrointestinal parasites may contribute to these conditions. As this study progressed, it was realized that *Cooperia punctata* frequently occurred in large numbers. Since this parasite has been implicated as being potentially harmful to cattle and causes scouring, anorexia, debilitation, and even death,[5, 9, 11, 12] attempts were made to find a suitable anthelmintic.

Some of the drugs which have been tried in more recent years for the treatment against gastrointestinal roundworms of cattle include phenothiazine, piperazine citrate, toluene, 1:8 dihydroxyanthraquinone, and four different organic phosphate compounds: ronnel* (0,0-dimethyl 0-[2,4,5-trichlorophenyl] phosphorothioate), Dowco 105** (0-methyl 0-[4-tert. butyl-2-chlorophenyl] ethylphosphophoramidothiate), Bayer L13/59† (0,0-dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate), and Bayer 21/199† (0,0, diethyl 0-[3-chloro-4-methyl-7-coumarinyl] phosphorothioate).

Swanson *et al.*[13] reported that nonconditioned phenothiazine at the rate of 0.4 to 1.1 Gm. per kilogram of body weight was slightly effective against Cooperia spp. (1.0 to 11.1%) and more effective against *Haemonchus contortus* (100%), *Ostertagia ostertagi* (78.8 to 96.6%), *Trichostrongylus axei* (100%), *Bunostomum phlebotomum* (23.7 to 100%), and *Oesophagostomum radiatum* (99.5 to 100%).

Swanson *et al.*[10] found that piperazine citrate at the rate of 14.2 Gm. of base per 100 lb. was 97.6 per cent effective against Cooperia spp., 100 per cent effective against *Oe. radiatum*, and less effective against *H. contortus* (15.1%), *O. ostertagi* (71.9%), *T. axei* (2.1%), *B. phlebotomum* (33.9%), *Trichuris discolor* (11.6%), and Nematodirus sp. (55%). Riek and Keith[13] found that toluene emulsion at the rate of 10 ml./100 lb. reduced Cooperia egg count approximately 70 to 90 per cent, and a similar high reduction in the egg count of Haemonchus and Bunostomum. Riek and Keith[14] also found 1:8 dihydroxyanthraquinone active against Cooperia but ineffective against Haemonchus and Trichostrongylus.

Herlich and Johnson[7] reported that ronnel at the rate of 100 mg. per kilogram yielded percentages of efficacy against various species of nematodes as follows: *C. punctata*, 59; *Haemonchus placei*, 98; *O. ostertagi*, 46; *Oe. radiatum*, 30; and Trichuris spp., 9 per cent. The drug was effective against *T. axei*, *Trichostrongylus colubriformis*, and *Nematodirus helvetianus*.

Baker *et al.*[3] reported that Dowco 105 at the rate of 120 to 130 mg. per kilogram yielded the following percentage efficacy: Cooperia spp., 87 per cent, *T. axei*, 100 per cent, *Trichostrongylus capricola* and *Trichostrongylus vitrinus*, 96 to 100 per cent, and *O. ostertagi*, 66 per cent.

Riek and Keith[15] found that Bayer L13/59 at the rate of 5 Gm./100 lb. reduced the egg count of *C. punctata*, *Cooperia pectinata*, and *T. axei* approximately 80 to 100 per cent. A dosage rate of 2 Gm./100 lb. yielded a high reduction of egg count for Haemonchus and Oesophagostomum.

Herlich and Porter[8] reported that Bayer 21/199 was 100 per cent effective against cooperids at the rate of 12.5 to 25.0 mg. per kilogram. It was also highly effective against Haemonchus (100%), Ostertagia (97.2%), Trichostrongylus (99.7%), Trichuris (96.0%), Oesophagostomum (100%), and Strongyloides.

This is a report of the incidence and abundance of helminths recovered from four groups of 4-, 8-, and 12-month-old beef and dairy calves which had been raised under normal conditions in areas of different rainfall. In addition, a report is made of results of critical tests conducted with

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* Trolene, produced by Dow Chemical Co., Midland, Mich.

** Produced by Dow Chemical Co., Midland, Mich.

† Available from the Chemagro Corp., New York, N.Y.

recent available drugs to determine their efficacy against *Cooperia punctata*.

MATERIALS AND METHODS

Fifteen bull calves, approximately the same age, were reserved for this study from each of two beef ranches and two dairies. The location and average annual rainfall of these areas are: ranch A (Kipu), Island of Kauai, rainfall 72 inches; ranch B, (Kaalualu), Island of Hawaii, rainfall 45 inches: dairy A (Kawailoa), Island of Oahu, rainfall 29 inches; dairy B (Waimea), Island of Kauai, rainfall 19 inches. In each area, the reserved calves were raised normally with other members of the same herd but were eventually slaughtered in groups of 5 when they reached 4, 8, and 12 months of age. At slaughter, the abomasums and intestines of these animals were examined for parasites as outlined by Porter.[10] The lungs and livers were also examined for lungworms and liver flukes, respectively.

In experiments involving treatment of calves, toluene, piperazine citrate, ronnel, and Bayer L13/59 were critically tested in recently weaned bull calves, approximately 2 to 3 months old. In addition, piperazine dihydrochloride* and a new organic phosphate compound, Ruelene** (4-tert. butyl-2-chlorophenyl methyl methylphosphoramidate), were used. Each calf was experimentally infected with 50,000 to 80,000 infective *C. punctata* larvae. About three weeks later, the infected calves were given the drug in an emulsion form as a drench. The efficacy of the drug was determined from the number of parasites eliminated with the feces for three days following treatment, and the number recovered at necropsy four days after treatment. For estimating the number of parasites found, the method described by Swanson *et al.*[18] was adopted.

* Dowzene DHC, produced by Dow Chemical Co., Midland, Mich.

** Produced by Dow Chemical Co., Midland, Mich.

RESULTS AND DISCUSSION

Parasite Findings.—In this study, a total of 60 calves from four areas in the Islands was examined. Fifteen were from each of two beef ranches and 15 were from each of two dairies.

Among beef calves (table 1), the total number of parasites found was generally higher on ranch A with an average rainfall of 72 inches than on ranch B with a rainfall of 45 inches. Stomach worms were generally few in all the calves. Haemonchus was absent in ranch A calves and present in ranch B calves (range, 0 to 60). Ostertagia ranged from 0 to 320, and Trichostrongylus from 0 to 1,040 in animals of both ranches. These worms were more

TABLE 1—Total Number (Range in Parentheses) of Parasites Found Among 4-, 8-, and 12-Month-Old Beef Calves (5 in Each Age Group) from Each of Two Ranches of Different Annual Rainfall

Location	Parasite	Age (months) 4	8	12
RANCH A (KIPU). AVERAGE ANNUAL RAINFALL, 72 INCHES				
Abomasum	*O. ostertagi*	320(20–120)	500(80–160)	880(40–320)
	T. axei	240(40–60)	260(20–80)	480(20–180)
Small intestine	*C. punctata*	2,980(380–900)	148,622(10,800–47,060)	25,300(160–9,840)
	S. papillosus	1,360(140–420)	220(40–120)	40(0–20)
	C. spatulata	220(0–100)	368(0–368)	0
	B. phlebotomum	80(0–55)	104(1–57)	91(0–61)
	Moniezia spp.*	50(8–14)	2(0–2)	0
Large intestine	*Oe. radiatum*	357(42–92)	303(32–98)	202(10–112)
	Trichuris spp.	0	2(0–2)	0
Lungs	*D. viviparus*	0	(1 positive)	0
Liver	*F. gigantica*	0	0	(1 positive)
RANCH B (KAALUALU). AVERAGE ANNUAL RAINFALL, 45 INCHES				
Abomasum	*T. axei*	100(0–60)	360(40–120)	2,600(140–1,040)
	O. ostertagi	20(0–20)	280(20–80)	700(60–300)
	H. placei	87(0–60)	144(15–42)	31(0–20)
Small intestine	*C. punctata*	960(20–600)	1,140(200–400)	580(40–220)
	S. papillosus	620(60–240)	180(20–60)	20(0–20)
	B. phlebotomum	45(1–17)	0	6(0–6)
	Moniezia spp.	36(2–13)	3(0–2)	4(0–3)
	C. spatulata	20(0–20)	0	0
Large intestine	*Oe. radiatum*	19(0–10)	14(0–5)	0
	Trichuris spp.	5(0–5)	0	0
Lungs	*D. viviparus*	0	0	0
Liver	*F. gigantica*	0	0	0

* Of 25 tapeworms (segments) identified, all proved to be *Moniezia benedeni*.

numerous in calves 12 months old. Of the roundworms of the small intestine, the number of *C. punctata* was greater than any other parasite, and most abundant in calves 8 months old. Calves 12 months old showed reduced cooperid infection, possibly due to acquired host resistance.[4,17] The number of Cooperia ranged from 160 to 47,060 in calves from ranch A and 20 to 600 in calves from ranch B. Infection with other intestinal helminths in all the beef calves was generally low. This included *Cooperia spatulata* (range, 0 to 368), Bunostomum (range, 0 to 61), Strongyloides (range, 0 to 420), Moniezia (range, 0 to 14), Trichuris (range, 0 to 5), and Oesophagostomum (range, 0 to 112). Light infections with lungworms and liver flukes were found in 2 calves from ranch A.

Among the dairy calves, the incidence and degree of infection was somewhat higher in dairy A, which has an average rainfall of 29 inches, than in dairy B, which has a rainfall of 19 inches. Of the stomach worms, a few Trichostrongylus (range, 20 to 60) were found only in 12-month-old calves in dairy B. Light infections with Haemonchus (range, 0 to 120) and Ostertagia (range, 0 to 140) were found in most calves of dairy A. Of the roundworms of the small intestine, *C. punctata* occurred in larger number than any others. In dairy A, cooperids were more numerous in 4-month-old calves (range, 1,540 to 51,380) than in older calves; the number ranged from 140 to 21,160 in the 8-month-old animals and 0 to 1,980 in those 12 months old. In dairy B, cooperids were more numerous in 8-month-old calves (range, 260 to 20,100) than the 4-month-old (range, 60 to 420) or 12-month-old calves (range, 2,040 to 8,400). A few Bunostomum occurred in only 1 of the 12-month-old calves of dairy A. The incidence of Strongyloides was low in all the dairy calves (range, 0 to 160, with higher numbers in the 4-month-old animals). Moniezia infection was low (range, 0 to 7) in all the 4- and 12-month-old calves and higher (range, 0 to 26) in 8-month-old calves. No lungworms were found in the dairy calves, but liver flukes were found in the 8- and 12-month-old calves of dairy B.

These data indicate that *C. punctata* is the most common and most numerous helminth of beef and dairy calves in this region. In beef cattle, the infection rate was high in areas of high rainfall and in animals about 8 months old. The fact that beef calves range in wide areas where the parasite larvae are not likely to be concentrated, and that calves do not graze extensively during their first few months of life, are

TABLE 2—Total Number (Range in Parentheses) of Parasites Found Among 4-, 8-, and 12-Month-Old Dairy Calves (5 in Each Age Group) from Each of Two Dairies of Different Annual Rainfall

Location	Parasite	Age (months) 4	8	12
DAIRY A (KAWAILOA). AVERAGE ANNUAL RAINFALL, 29 INCHES				
Abomasum	*H. placei*	46(0–26)	380(20–120)	157(18–83)
	O. ostertagi	0	60(0–40)	340(20–140)
Small intestine	*C. punctata*	86,520(1,540–51,380)	32,060(140–21,160)	2,000(0–1,980)
	C. spatulata	1,092(0–758)	0	0
	S. papillosus	580(20–160)	100(0–40)	0
	Moniezia spp.	2(0–2)	3(0–3)	0
	B. phlebotomum	0	0	3(0–3)
Large intestine	Trichuris spp.	168(6–74)	15(0–15)	0
	Oe. radiatum	42(0–15)	35(0–12)	25(0–13)
Lungs	*D. viviparus*	0	0	0
Liver	*F. gigantica*	0	0	0
DAIRY B (WAIMEA). AVERAGE ANNUAL RAINFALL, 19 INCHES				
Abomasum	*T. axei*	0	0	180(20–60)
Small intestine	*C. punctata*	1,320(60–420)	36,200(260–20,100)	22,880(2,040–8,400)
	S. papillosus	240(20–80)	0	0
	Moniezia spp.	1(0–1)	68(2–26)	7(0–7)
Large intestine	Trichuris spp.	335(47–92)	148(15–52)	0
Lungs	*D. viviparus*	0	0	0
Liver	*F. gigantica*	0	(5 positive)	(5 positive)

factors which may account for the usual low parasitism at 4 months of age. As they grow older and progressively graze more, opportunity for ingesting infective parasite larvae with grass increases. Thus, heavy parasitism may be encountered among calves 8 months old. However, young dairy calves, which under local conditions are frequently kept in small grassy paddocks used year after year, are likely to become exposed to heavy infection early in life. Heavy parasitism may be expected in these calves at 4 months of age or even younger, especially in areas of high rainfall.

In connection with the incidence and degree of parasites in calves, Roberts *et al.*[16] have reported in Australia that Cooperia sp. builds up much more rapidly and at younger age than infection with other trichostrongyles. They also observed that, although larvae of Trichostrongylus and Ostertagia were detected in fecal samples of calves 6 to 8 months old, in many animals evidence of infection with these species was frequently delayed. The present observations are in accord with those of the Australian workers.

The incidence of helminths found among all the calves examined is summarized (table 3). In addition, a few coccidia oocysts were found in the feces of several of these calves.

TABLE 3—Internal Parasites Recovered at Necropsy from 60 Calves, 4 to 12 Months Old, Arranged in Accordance to Their Percentage of Incidence

Parasite	Location	No. of animals infected	Per cent parasitized
C. punctata	Small intestine	57	95.0
Oe. radiatum	Large intestine	37	61.6
S. papillosus	Small intestine	34	56.7
O. ostertagi	Abomasum	33	55.6
T. axei	Abomasum	33	55.6
Moniezia spp.	Small intestine	25	41.6
B. phlebotomum	Large intestine	24	40.0
H. placei	Abomasum	23	38.3
Trichuris spp.	Large intestine	18	30.0
F. gigantica	Liver	11	18.3
C. spatulata	Small intestine	8	13.3
D. viviparus	Lungs	1	1.6

Efficacy of Various Drugs.—The findings in this study indicate that *C. punctata* primarily, and to a lesser extent other species of parasites, are responsible for or contribute to the unthriftiness in some of the local calves. The problem appears to be more acute among dairy calves maintained in small grassy paddocks used constantly, and among beef calves raised in areas of high rainfall. In these areas, it would seem desirable to treat beef calves for Cooperia at 7 to 8 months of age and to treat dairy calves at 4 to 8 months of age as needed. In this connection, increased body weight has been noticed by the writer[1] in the treatment of three unselected groups of 6-month-old calves which were treated with piperazine dihydrochloride at the rate of 20 Gm. of base per 100 lb. of body weight. The calves had moderate Cooperia infections and light infections with Bunostomum and Strongyloides. The average daily body gains of these animals for 90 days following treatment were: treated calves, 1.24 lb. (group 1, 30 beef calves), 1.21 lb. (group 2, 15 dairy calves), and 0.72 lb. (group 3, 25 beef calves). For the corresponding untreated control calves, the gains were 1.09 lb., 0.98 lb., and 0.58 lb., respectively.

The results obtained in the present study of the treatment of calves against *C. punctata* are summarized (table 4). Toluene and phenothiazine were found ineffective in 3 calves treated. Piperazine citrate at the rate of 15 Gm. of base per 100 lb. was 89.3 to 95.6 (av., 92.4) per cent effective in 2 calves treated. Its high efficacy confirms the report of Swanson *et al.*[19] Piperazine dihydrochloride at the rate of 20 Gm. of base per 100 lb. was 78.6 to 99.0 (av., 87.8) per cent effective in 5 animals treated. Ronnel bolus administered to 6 calves at the rate of 5 Gm. (active ingredient) per 100 lb. was 42.8 to 99.7 (av., 79.7) per cent effective. In these trials, the efficacy of ronnel against cooperids was a little higher than that reported by Herlich and Johnson.[7] Bayer L13/59 administered to 5 calves at the rate of 3.0 Gm. (active ingredient) per 100 lb. was from 67.1 to 100 (av., 88.8) per cent effective. Ruelene was found to be 98.9 to 100 (av., 99.7) per cent effective in 6 calves which were treated at the rate of 1.76 Gm. (active ingredient) per 100 lb. The high efficacy of this drug against Cooperia appears to be similar to that of Bayer 21/199 as reported by Herlich and Porter.[8]

Of these drugs, the piperazines and most of the organic phosphates appeared to be highly effective against *C. punctata*. However, toxicity is an important factor to consider in connection with the practical use of these drugs. The piperazine salts seem to be well tolerated by cattle. Swanson *et al.*[19] found no toxicosis in 10 calves treated

with piperazine citrate. Similarly, no ill effects were seen in 11 calves treated with piperazine citrate and piperazine dihydrochloride in this study, and in 70 additional 6-month-old beef calves treated under field conditions.[1]

Some of the organic phosphate compounds, however, are not well tolerated by cattle. Baker *et al.*[3] reported toxicosis in 5 of 10 calves treated with Dowco 105. Herlich and Porter[8] observed mild and transitory signs of intoxication in calves treated with Bayer 21/199 and suggested that the 25 mg. per kilogram level probably should not be exceeded. In the use of this drug, and possibly other related compounds, Douglas *et al.*[6] stated that the incidence of intoxication resulting from cholinesterase inhibition cannot be predicted with accuracy. They suggested that more information is needed to understand this problem. Riek and Keith[15] saw no clinical manifestation of toxicity among calves treated with Bayer L13/59 at the rate of up to 5 Gm./100 lb. However, in this study, 2 calves (weighing 232 and 257 lb., respectively) treated with Bayer L13/59 at this dosage rate showed inappetence and slight dragging of the hind feet on the day of treatment. When this drug was administered to 5 other calves at the rate of 3 Gm./100 lb. (table 4), it produced diarrhea for two days in the 4 calves weighing 118 to 150 lb. One of these calves, weighing 248 lb., showed inappetence for two days but had no evidence of toxicosis.

In contrast with the previously mentioned organic phosphates, Ruelene* at the rate of 1.76 Gm./100 lb. (39 mg./kg.) produced no evidence of toxicosis in 6 of the calves treated (table 4). In addition, toxicosis did not affect 7 other calves similarly treated under field conditions. These animals weighed 234 to 436 lb. (av., 298.4 lb). It appears, therefore, that this drug shows promise of being safe and highly efficient against *C. punctata* and possibly other intestinal parasites of cattle. Information furnished by the producers of this drug

TABLE 4—Efficacy of Various Drugs Against Cooperia punctata in Young Cattle

Drug used	Animal weight (lb.)	Dosage rate for 100 lb. body weight (In grams, except as indicated)	Worms eliminated with feces	Worms recovered at necropsy	Efficacy (%)	Average efficacy (%)
Toluene	125	10 cc.	0	4,900	0	0
	120	10 cc.	0	31,000	0	0
Phenothiazine	110	20.0	0	7,040	0	0
Piperazine citrate	105	10.8*	26,400	3,780	87.5	
	104	10.8	4,844	1,646-	74.6	81.1
	139	15.0	5,380	250	95.6	
	133	15.0	2,000	240	89.3	92.4
Piperazine dihydrochloride	120	15.0*	4,680	6,460	42.0	
	128	15.0	13,530	6,080	68.9	54.5
	108	20.0	8,120	2,200	78.6	
	105	20.0	12,200	120	99.0	
	105	20.0	10,530	1,080	90.7	
	110	20.0	16,120	2,020	88.9	
	115	20.0	22,380	5,040	81.6	87.8
Ronnel	110	5.0**	14,900	1,425	91.3	
	120	5.0	16,875	1,350	92.6	
	124	5.0	12,050	13,720	42.8	
	120	5.0	16,090	10,580	60.3	
	108	5.0	34,920	120	99.7	
	135	5.0	51,460	4,680	91.7	79.7
Bayer L13/59	118	3.0**	18,750	0	100.0	
	138	3.0	33,140	120	99.6	
	133	3.0	21,320	0	100.0	
	150	3.0	20,080	9,840	67.1	
	248	3.0	7,020	2,020	77.7	88.8
Ruelene	110	1.76**	43,930	0	100.0	
	246	1.76	22,450	260	98.9	
	148	1.76	24,240	0	100.0	
	133	1.76	36,150	0	100.0	
	168	1.76	36,450	0	100.0	
	148	1.76	25,500	0	100.0	99.7

* Amount of piperazine base. ** Amount of active ingredient.

states that Ruelene is active against Trichostrongylus, Haemonchus, Ostertagia, Cooperia, and Oesophagostomum. It also states that unfavorable side effects in cattle have occasionally been noticed with this drug at levels of 30 to 50 mg. per kilogram.

Comparing the usefulness of the piperazines and Ruelene, the latter is more effective against Cooperia and a smaller quantity is required for treatment. However, in view of the unfavorable side effects commonly ascribed to organic phosphate compounds, it appears that further field trials with Ruelene with respect to its safety are desirable before definite recommendations for its use can be made.

SUMMARY

Report is made of helminths found in the internal organs of beef and dairy calves (2 groups of each) raised under natural conditions in areas of different annual rainfall. *Cooperia punctata* was found to be more numerous than any other helminth present and was most prevalent in areas of high rainfall. The high incidence and abundance of this parasite, and to a lesser extent the presence of other species of parasites, appear to be contributing factors to the unthriftiness of some of the local calves.

Of two groups of beef calves examined, the number of Cooperia was greater in those 8 months old than in those 4 or 12 months old. Of the two groups of dairy calves, Cooperia infection was highest in 4-month-old animals in one group, and 8-month-old animals in the other group. It appears that beef calves raised in wide areas with their dams acquire the infection more slowly than dairy calves kept in close confinement. It is suggested that beef calves be given anthelmintic treatment against Cooperia at the age of 7 to 8 months and dairy calves at the age of 4 to 8 months, as needed.

Various drugs, including phenothiazine, toluene, piperazine citrate, piperazine dihydrochloride, and three organic phosphate compounds (ronnel, Bayer L13/59, and Ruelene) were critically tested against experimental *Cooperia punctata* infections in calves. Of these, the piperazines and Ruelene yielded better results with respect to combined efficacy against the parasite and tolerance of the animals to the drug. Of these two, Ruelene was found to be more effective. A dosage rate of 1.76 Gm./100 lb. of body weight gave an over-all anthelmintic efficacy of 99.7 per cent.

* Produced by Dow Chemical Co., Midland, Mich.

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Chemotherapeutic Studies of Gastrointestinal Parasites of Beef Cattle and Bison in Kansas

D. E. WORLEY, Ph.D., and M. F. HANSEN, Ph.D.

Manhattan, Kansas

PRESENT TREATMENT of gastrointestinal helminthiasis in cattle is contingent upon the action of a limited number of anthelmintic compounds which are known to be ineffective against certain species of bovine parasites. While extensive research has indicated phenothiazine to be the preferred drug for ruminant nematodes, critical testing in cattle has demonstrated that it is relatively ineffective against Cooperia, Bunostomum, and some species of Trichostrongylus and Ostertagia.[7, 21] Likewise Trichuris, Strongyloides, and cestodes of the genus Moniezia [20] are not affected by phenothiazine. Furthermore, it is not active against larval stages of any of the stomach and intestinal worms of cattle.[5] The limited effectiveness of the compound when administered to cattle as a prophylactic agent at low levels in feed further restricts its utility. Extensive studies on the free-choice feeding of small quantities of the drug [2, 3, 12, 13] have indicated that, while it definitely curtails nematode egg production in treated cattle, prophylactic phenothiazine levels are too low to remove worms, and consumption of medicated feed is often erratic.

The foregoing studies revealed the need for additional anthelmintics to supplement phenothiazine to control worm infections in cattle. The purpose of the present trials was twofold: First, several antibiotics and hormones now used principally as feed supplements were administered concurrently with phenothiazine to determine whether combined dosing with these substances might condition or perhaps potentiate anthelmintic activity of phenothiazine; second, the study investigated the antiparasitic activity of two organic phosphate compounds, ronnel (Dow-ET-57)* and Dowco 109.**

MATERIALS AND METHODS

In most instances, drugs were administered routinely as powdered feed additives at predetermined dose levels; in some tests, the organic phosphates were administered orally as a bolus. Otherwise, drug consumption by each group of cattle was determined by weighing uneaten feed at the end of the treatment period.

Quantitative determination of the number of helminth eggs per gram of feces (e.p.g.) was used to estimate anthelmintic activity. The principle of fecal egg-counting procedures for determining relative levels of gastrointestinal parasitism in ruminants has been verified statistically by Krug and Mayhew [9] and Spedding.[19] The reliability of such techniques when an adequate number of animals are utilized has been affirmed by Roberts *et al.*[17, 18] Similar methods for evaluating the activity of anthelmintics in cattle have been used by Porter *et al.*,[16] Cauthen,[3, 4] and Mayhew.[13, 14]

A modified Lane sodium nitrate flotation technique [10] was used in preparing fecal samples for egg count determinations. Differential nematode ova counts were prepared by grouping them into six categories according to their shape, size, shell characteristics, and appearance of blastomeres. Since it was impossible to distinguish the genus of certain parasites by this technique, evidence of drug activity usually was based on the total, rather than the differential, egg count of an animal. Individual counts were prepared from numbered animals at regular intervals during the tests, thus providing a periodic indication of the course of infection of each animal. Pretreatment counts were followed at intervals (usually monthly) by additional counts made during or after the planned course of treatment or both. A comparison of pre- and post-treatment e.p.g. was used to evaluate drug activity against the parasites.

Infections with the nematode genera Cooperia, Trichostrongylus, and Ostertagia (counted collec-

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* 0,0-dimethyl 0-2, 4,5-trichlorophenyl phosphorothioate, produced as Trolene by the Dow Chemical Co., Midland, Mich.

** 0-4 (tert. butyl-2-chlorophenyl) 0-methyl methyl phosphoramidothioate is produced by the Dow Chemical Co., Midland, Mich.

tively) ordinarily predominated in the test cattle and bison, while Haemonchus and Oesophagostomum also occurred frequently. Occasional infections with Nematodirus, Bunostomum, Trichuris, Strongyloides, and the cestode Moniezia were encountered in a low percentage of the cattle. All infections were acquired naturally, and usually fell within the range of 0 to 150 e.p.g.

Hereford cattle were used exclusively as test animals, except in experiment 6 which utilized bison from the herd of the Kansas Forestry, Fish and Game Commission at Garden City. In experiment 2, heifers were used and in experiment 6, bison bulls, cows, and calves. All other experiments were made with steers. Each cattle experiment was limited to animals of comparable condition and approximately equal age. Periodic weights, acceptance of medicated feeds, and behavior following treatment were used as criteria of drug toxicity to the host.

Statistical analyses were performed when applicable to aid in evaluating the experimental data. The word "significant" in this study has a statistical connotation. Analyses of variance were used routinely to compare e.p.g. in the anthelmintic tests. When pretreatment counts were not normally distributed, they were transformed by using the square root of x plus 1. In one case, the one-sided U test of Mann and Whitney [11] was used in a nonparametric test of the data for significance.

RESULTS

The experimental design and results of individual and combined treatment with phenothiazine, chlortetracycline, and diethylstilbestrol are summarized (table 1).

Experiment 1.—Each of the eight treatment regimens were replicated in two lots of 5 animals each. The anthelmintic action of both therapeutic and continuous low-level phenothiazine; continuous chlortetracycline feeding at 75.0 mg./day and 0.5 mg./lb./day; low-level stilbestrol at 10.0 mg./day; and various phenothiazine, chlortetracycline, and stilbestrol combinations were tested in 650-lb. feeder steers. Although none of the treatments significantly reduced the number of worm eggs in the feces, chlortetracycline at both levels tested produced the most immediate as well as the most prolonged reduction in e.p.g. Low-level phenothiazine at 2 Gm./day had no depressant effect on e.p.g. levels. Weight gains in animals receiving chlortetracycline, stilbestrol, and phenothiazine concurrently were significantly greater than those of the controls. None of the drug regimens reduced feed palatability or produced any visible signs of toxicosis in the test animals.

Experiment 2.—The effect of daily dosing with 75 mg. of chlortetracycline alone or in combination with various regimens of phenothiazine was tested in 24 heifers (av. wt. 420 lb.) over a 10½-month period (table 1). A significant reduction in worm egg counts occurred among heifers receiving chlortetracycline plus 2 Gm. of phenothiazine per day and among those receiving chlortetracycline plus 12 Gm. of phenothiazine per day for four days followed by 2 Gm. per day for 10½ months. Both of these treatments were shown to be consistently effective at all monthly sampling dates. Weight gained by cattle in all treated groups was superior, but not significantly greater than that of the controls.

Experiment 3.—In this test, the effects of phenothiazine at therapeutic levels followed by daily low-level phenothiazine for eight months were studied in 5 steers (av. wt. 420 lb.). Neither the initial dose nor the subsequent 2-Gm. daily doses affected the number of worm eggs passed by the treated cattle. Weight gains of the treated and control animals were essentially alike.

Experiment 4.—The effects of a single dose of ronnel were evaluated in 38 yearling cattle (av. wt. 400 lb.) during a five-month test. The cattle were separated into seven lots, each lot containing treated and control animals. To compare the efficacy of the bolus with feed formulations of the compound, half of the treated animals received boluses and half received equivalent drug in feed.

Worm egg counts were determined prior to treatment, at one and two weeks post-treatment, and at one, two, three, four, and five months post-treatment (table 2). One week after dosing, the e.p.g. counts of treated cattle had decreased 83.5 per cent. This reduced level of infection remained essentially constant during the remaining 4½ months of the experiment, rising to a peak of 20.1 per cent of the pretreatment count five months post-treatment. Average e.p.g. levels in the control cattle gradually decreased during the same period. Differences between treated and control counts at all post-treatment dates were significant ($P<0.05$). Both formulations were equally effective in depressing e.p.g. Neither treatment had any effect on weight gains of the recipient animals. Consumption of the medicated feed indicated its palatability was not reduced.

Experiment 5.—A confirmatory test designed to provide additional evidence of the anthelmintic potential of a single dose of ronnel was conducted in 36 yearling cattle of similar age and condition to those used in the preceding experiment. Twelve animals were treated by bolus at the rate of 110 mg./kg., and 12 received an equivalent dose in feed which was consumed in one day. A third group of 12 served as untreated controls. Monthly e.p.g. checks were made during a 90-day period extending from four to seven months after treatment. Although no pretreatment egg count data were available, random assortment of the test groups provided a statistical basis for comparing their respective worm burdens.

Irrespective of date or method of drug administration, the post-treatment e.p.g. counts of the treated cattle were significantly less than those of their controls (table 2). The greatest variance occurred at four months post-treatment, when counts of treated and control groups were 1.9 and 9.8 e.p.g., respectively, and decreased gradually during post-treatment. Final counts made seven months post-treatment averaged 5.8 e.p.g. (treated) as compared with 10.8 e.p.g. (controls). Weight gains of all cattle were similar.

Experiment 6.—Eighteen bison cows, calves, and young bulls were treated individually with ronnel at the rate of approximately 110 mg./kg.; the remaining animals in the herd served as controls. A single pretreatment fecal sample was collected, followed by post-treatment checks at 10-, 36-, and 60-day intervals (table 2).

From ten days and extending to five

TABLE 1—Effects of Individual and Combined Administration of Phenothiazine, Chlortetracycline, and Diethylstilbestrol on Fecal Egg Counts in Hereford Cattle

Experiment	No. of animals	Treatment	Dosage and duration	Eggs per gram of feces: Pretreatment (av.)	Initial count during treatment (av.)*	Change (%)	Final (av.)**	Change (%)
1	10	Control	.....	39.4	14.8	−62.4	71.1	+380.0
	10	Chlortetracycline	75.0 mg./day–4 mo.	43.5	18.6	−57.2	12.8	−31.1
	10	Diethylstilbestrol	10.0 mg./day–4 mo.	64.1	126.8	+97.8	59.8	−52.8
	10	Phenothiazine	2.0 Gm./day–4 mo.	25.8	55.6	+115.0	31.0	−44.2
	10	Chlortetracycline	0.5 mg./lb./day–4 mo.	36.9	30.8	−16.5	7.6	−75.3
	10	Chlortetracycline + Diethylstilbestrol	75.0 mg./day–4 mo. + 10.0 mg./day–4 mo.	20.0	48.8	+144.0	14.3	−70.6
	10	Chlortetracycline + Phenothiazine	75.0 mg./day–4 mo. + 2.0 Gm./day–4 mo.	47.2	48.3	+2.3	4.1	−91.5
	10	Diethylstilbestrol + Phenothiazine	10.0 mg./day–4 mo. + 2.0 Gm./day–4 mo.	29.5	39.3	+33.2	23.9	−39.1
	10	Chlortetracycline + Diethylstilbestrol + Phenothiazine	75.0 mg./day–4 mo. + 10.0 mg./day–4 mo. + 2.0 Gm./day–4 mo.	25.3	52.8	+108.0	77.1	+46.0
2	12	Control	.....	95.4	51.5	−46.0	104.2	+102.0
	6	Chlortetracycline	75.0 mg./day–10½ mo.	60.3	27 2	−54.8	90.7	+233.0
	6	Chlortetracycline + Phenothiazine	75.0 mg./day–10½ mo. + 2.0 Gm./day–10½ mo.	43.8	10.3†	−76.4	64.2†	+523.0
	6	Chlortetracycline + Phenothiazine	75.0 mg./day–10½ mo. + 12.0 Gm./day–4 days	71.8	33.3	−53.6	81.2	+143.0
	6	Chlortetracycline + Phenothiazine + Phenothiazine	75.0 mg./day–10½ mo. + 12.0 Gm./day–4 days + 2.0 Gm./day–10½ mo.	71.0	17.2†	−75.7	63.0†	+266.0
3	5	Control	.....	67.0	136.8	+104.0	63.4	−53.6
	5	Phenothiazine + Phenothiazine	12.0 Gm./day–4 days + 2.0 Gm./day–8 mo.	93.0	54.8	−41.0	46.5	−15.2

* Experiment 1, two months; experiment 2 and 3, one month; ** experiment 1, four months; experiment 2, 10½ months; experiment 3, six months; † $P<0.05$.

weeks post-treatment, the average e.p.g. counts of treated and untreated bison were significantly different. However, at 60 days post-treatment, the egg counts between the two groups were not significantly different.

Experiment 7.—The anthelmintic activity of Dowco 109, an experimental organic phosphate compound, was tested against gastrointestinal parasites in 40 yearling cattle weighing about 400 lb. in a four-week feedlot study. Worm egg counts were made before treatment and one, two, and four weeks post-treatment (table 2). None of the three treatment regimens, in which a total dose of 15 mg./kg. of drug was given in one, six, or 12 days, had any effect on internal parasites as evidenced by reduced e.p.g. No indication of gross toxicity was noticed.

TABLE 2—Effects of Two Organic Phosphate Compounds on Fecal Egg Counts of Bison and Hereford Cattle

Experiment	No. of animals	Treatment	Dosage and duration	Eggs per gram of feces: Pretreatment (av.)	1st post-treatment (av.)*	Change (%)	2nd post-treatment (av.)**	Change (%)
4	13	Control	.....	16.2	11.8	−27.2	11.5	−2.5
	25	Ronnel	110.0 mg./kg.–1 day	19.4	3.2†	−83.5	2.7†	−15.6
5	12	Control	.....	.....	9.8	.....	4.0	−59.1
	24	Ronnel	110.0 mg./kg.–1 day	.....	1.9†	.....	1.1†	−42.1
6	18	Control	.....	42.2	48.4	+14.7	13.1	−72.9
	18	Ronnel	110.0 mg./kg.–1 day	42.2	7.1†	−83.1	6.3†	−11.3
7	10	Control	.....	51.7	45.1	−12.8	44.6	−1.1
	10	Dowco 109	2.3 mg./day–6 days	59.3	49.5	−16.5	117.8	+138.0
	10	Dowco 109	1.2 mg./day–12 days	27.6	24.1	−12.7	109.3	+353.0
	10	Dowco 109	15.0 mg.–1 day	73.6	110.9	+50.3	130.0	+17.2

* Experiments 4, 6, 7, one week; experiment 5, four months; ** experiments 4, 7, four weeks; experiment 5, five months; experiment 6, five weeks; † $P<0.05$.

DISCUSSION

The anthelmintic utility in cattle of phenothiazine-supplemented feeds containing additional antibiotic or hormone additives has been compared under field conditions with the individual administration of phenothiazine, chlortetracycline, diethylstilbestrol, and two organic phosphate compounds. The relative merits of the various medications were assessed by comparing percentage reduction in post-treatment e.p.g. counts, acceptance of the drug as indicated by feed consumption and rate of gain, and nature and extent of any measurable side effects of treatment.

Of the seven composite treatments tested, continuous low-level feeding of chlortetracycline and phenothiazine was the only joint regimen that showed significant activity. The reduction in e.p.g. resulting from this diet may be attributed to the apparent synergistic action of the component compounds.

Why phenothiazine failed to depress worm egg counts, whether administered as a 48-Gm. (12 Gm./day–4 days) therapeutic dose, as a 2-Gm. daily dose, or as a combination of both regimens, is unknown. Although some difficulty was experienced with palatability of feed in administering the planned 60-Gm. therapeutic dose to cattle, each animal received approximately 48 Gm. of drug in each of two experiments. This intake may have been inadequate to act as an effective anthelmintic, although Britton [1] found that doses of 20 to 30 Gm. were sufficient to treat parasitic gastritis in yearling cattle. The 2-Gm. daily dose was consumed readily in all instances, but it was uniformly ineffective. Mayhew [13,14] found that daily doses of 1.5 Gm. and 0.5 Gm., respectively, were adequate to suppress ovulation by Haemonchus and Oesophagostomum completely within 14 days, and Herlich and Porter [6] reported effective control of Haemonchus and Oesophagostomum in pastured calves receiving 0–3 Gm. of a phenothiazine-mineral mixture daily by free-choice consumption. The general level of parasitism encountered in these experiments was entirely subclinical, thus somewhat artificially restricting the potential range of activity of the drug. Past work [3] has shown, however, that the effec-

tive use of phenothiazine has not been confined to treatment of severe clinical parasitism. A number of factors, including erratic consumption of medicated feed and individual variation in drug intake, due to the free-choice treatment system used, contributed to the poor performance of phenothiazine.

Ronnel was the only drug administered alone as a single dose which showed promising anthelmintic activity. Both the consistency and relative stability of post-treatment e.p.g. reductions indicated that a single 110-mg./kg. dose either acted directly as an anthelmintic or in some manner materially reduced worm egg production. Critical ronnel testing on a limited number of animals by Norris [15] showed that doses of 125 to 150 mg./kg. removed 100 per cent of Haemonchus, 88 to 95 per cent of Ostertagia, 81 to 98 per cent of Cooperia, and 27 to 38 per cent of Oesophagostomum; daily doses as low as 5 mg./kg. were sufficient to suppress nematode ovulation while retaining mild anthelmintic activity. Herlich and Johnson [8] reported that ronnel at a level of 110 mg./kg. gave the following percentage reductions in worm burden in cattle: *Haemonchus placei,* 98.0; *Ostertagia ostertagi,* 46.0; *Trichostrongylus axei,* 0.4; *Cooperia punctata,* 59.0; *Oesophagostomum radiatum,* 30.0; and Trichuris sp., 9.0 per cent.

In view of the foregoing critical data, at least a part of the marked e.p.g. reduction observed in the present experiments presumably was indicative of a reduced worm burden in the treated cattle. Only in the bison study did the post-treatment e.p.g. count recover to approximately the pretreatment level within 60 days. Here the conditions under which the animals were grazed and watered on the range permitted constant exposure to reinfection, so the observed increase may be attributed tentatively to patent reinfections. Although the nature of the anthelmintic action of ronnel in cattle is unknown, the consistent effectiveness of a single dose in controlling e.p.g., coupled with the ease of diet administration, the associated benefits of general insect protection, and the absence of gross toxic side effects, recommend the compound for further evaluation as a bovine parasiticide.

SUMMARY

Field studies are reported of the anthelmintic activity of five drugs in 13 different combinations which were administered primarily as feed additives to bison and Hereford cattle. Worm egg counts of heifers receiving 75 mg. of chlortetracycline plus 2 Gm. of phenothiazine per day, regardless of whether preceded by a therapeutic dose of 48 Gm. of phenothiazine, were significantly lower than their controls. Reduced e.p.g. levels persisted during the 10½-month treatment period. No measurable anthelmintic activity was attributed to chlortetracycline, phenothiazine, diethylstilbestrol, or Dowco 109 when these compounds were administered separately. A single 110-mg./kg. dose of ronnel in either bolus or feed formulation significantly reduced e.p.g. in feedlot cattle for five and seven months, respectively. In range bison, appreciably reduced e.p.g. persisted for 36 days following ronnel treatment, but returned to pretreatment levels by 60 days post-treatment.

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Isolation and Identification of *Leptospira hardjo* from Cattle in Louisiana

E. E. ROTH, D.V.M., and MILDRED M. GALTON, Sc.M.

Baton Rouge, Louisiana

THE PRINCIPAL etiologic agent of bovine leptospirosis in the United States is presently considered to be *Leptospira pomona*. Within the last two years, attention has been brought to *Leptospira canicola*[14] infections in cattle with clinical manifestations of disease. Galton *et al.*[7] in 1952 reported the natural occurrence of agglutinins in cattle serums tested with antigen prepared from *Leptospira sejroe*, a member of the hebdomadis serogroup. Subsequently, antibodies against *L. sejroe* have been found frequently in cattle serums in Virginia,[10] Maryland,[2] Illinois,[4] Georgia,[9] Wisconsin, Oregon,[15] and many other states. To the authors' knowledge, previous attempts to isolate leptospires responsible for these antibodies have been unsuccessful.

The purpose of this report is to describe the isolation and identification of *Leptospira hardjo*, another member of the hebdomadis serogroup, from cattle in Louisiana, and to show the close antigenic relationship of *L. hardjo* to *L. sejroe*. Additional studies will be reported in subsequent papers.

MATERIALS AND METHODS

The cattle herd (H-herd) investigated consisted of 288 registered Herefords maintained at Chambers, La., at the Dean Lee Agricultural Center, a branch of the Louisiana State University. The calf crops for the previous five-year period were considered normal and no clinical signs of disease were noted by the husbandman. However, as leptospirosis due to *L. pomona* had been diagnosed in an adjacent herd, serum samples were collected from H-herd in June, 1957, to determine whether leptospiral antibodies were present. The serums were examined by the plate-agglutination test with *L. pomona* and *L. sejroe* antigens since antibodies to both of these serotypes had been detected in cattle in Louisiana. Selected samples were tested by the microscopic-agglutination test with live antigens of these two serotypes.

Macroscopic leptospiral slide-agglutination test antigens were prepared according to the method of Galton *et al.*,[6] with slight modification in standardization and application. The density was adjusted to 30 per cent light transmission on a Bausch and Lomb photoelectric colorimeter, (spectronic 20), at a wavelength of 550 mμ. *Leptospira pomona* (S-91) and *L. sejroe* (Mallersdorf) cultures were used to prepare the antigens.

Serums to be tested were diluted with sterile 0.85 per cent sodium chloride solution so that 0.04 ml. of each serum dilution mixed with 0.04 ml. of the respective antigen on a scored glass plate provided final dilutions of 1:10, 1:40, 1:160, and 1:640. The serum and antigen were mixed with a toothpick and rotated on a mechanical rotator (with speed regulator) for five minutes at 150 r.p.m. The plate was removed, rotated slowly by hand for 30 seconds and the reactions read against a black background. Degree of agglutination was recorded as positive if definite clumping occurred, as negative if no agglutination occurred.

On the basis of preliminary positive serologic findings, 2 bulls, M-592 and M-596, approximately 1 year old, from H-herd were selected for further study. They were transported to the research facilities of the Department of Veterinary Science, Louisiana State University. Three normal Jersey bull calves, C-26, C-31, and C-33, about 8 months old, were selected for experimental infection. Serums obtained prior to inoculation did not have antibodies for *L. sejroe* or *L. pomona* or when tested in retrospect, to *L. hardjo*. Within five minutes, 20 ml. of urine collected from calf M-592 was inoculated intravenously into C-26. Two hours later, this procedure was repeated with 20 ml. of urine collected from M-596. On the 48th day after inoculation, urine was collected from C-26 and 20 ml. inoculated intravenously into calf C-31; on the 61st day, urine was obtained from C-26 and inoculated intravenously into calf C-33 (table 1). Body temperatures of the calves were recorded daily from the first to the eighteenth day post-inoculation. Blood was collected periodically from C-26 for six months; from C-31 for five months; from C-33 for four months, and tested for leptospiral antibodies.

From the Department of Veterinary Science and the Agricultural Experiment Station, Louisiana State University, Baton Rouge.

From the Department of Health, Education and Welfare, Public Health Service, Bureau of State Services, Communicable Disease Center, Chamblee, Ga.

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TABLE 1—Summary of Data on Experimental Calves

Data	Calf 26	Calf 31	Calf 33
Inoculum	20 ml. urine from calf M-592 and 20 ml. urine from M-596 intravenously 2 hours apart.	20 ml. urine from calf 26 intravenously.	20 ml. urine from calf 26 intravenously.
Date inoculated	Aug. 16, 1957	Oct. 3, 1957	Oct. 16, 1957
Signs of disease	Moderate fever 9th day after inoculation.	None detected. Temperature taken daily for 18 days after inoculation.	None detected. Temperature taken daily for 18 days after inoculation.
Hemoculture results	None taken.	Leptospires isolated from all tubes of Stuart's semisolid on 9th day. All Fletcher's tubes neg. (only day cultured).	Leptospires isolated in all tubes of Stuart's semisolid on the 8th, 9th, 10th, and 11th days. All Fletcher's tubes neg. (daily cultures days 6–12).

Serologic results, see table 4.

Stuart's medium [12] was modified by the addition of 0.3 per cent nutrient agar (Difco No. 0001). This medium was used with Fletcher's medium [5] for comparison on all cultures from the inoculated calves. Both mediums were dispensed in 18- by 125-mm. screwcap tubes in 10- to 12-ml. aliquots and tested for sterility and ability to support growth of leptospires before use.

Isolation of leptospires from the blood of C-31 was attempted on the ninth day after inoculation and from C-33 on days 6, 7, 8, 9, 10, 11, and 12. The blood was obtained from the jugular vein with sterile 2.0-ml. syringe and three to five drops was inoculated immediately into each of four tubes of modified Stuart's and four tubes of Fletcher's mediums. The cultures were incubated at 30 C. for 60 days and examined periodically by dark ground microscopy before discarding.

Microscopic Agglutination and Agglutinin-Absorption Procedures.—Immune serums were prepared according to previously described methods.[8]

Leptospiral strains used for antigens in the microscopic-agglutination tests were maintained in Stuart's liquid medium and transferred every five days. The antigens consisted of five- to seven-day-old cultures in Stuart's medium. Before use, cultures were examined by dark ground microscopy for density and smoothness. If the cultures appeared too dense, they were diluted with sterile Stuart's medium. Antigen for *L. hardjo* strain LSU 91 was five- to seven-day-old cultures grown in Chang's [3] liquid medium. Leptospiral strains used in this study were obtained from the Leptospira Reference Laboratory, Walter Reed Army Institute of Research, Washington, D.C., except *L. kremastos*, which was obtained from the Leptospira Reference Laboratory, Brisbane, Australia, and *L. mini*, AB Sari, from the Instituto Superiore de Sanita, Rome, Italy. The strain designation, LT 117, is given to an unclassified member of the hebdomadis serogroup.

In the cross-agglutination studies on strain LSU 91, serial twofold dilutions were prepared in buffered 0.85 per cent sodium chloride solution to provide serum dilutions of 1:25 through 1:51,200. To 0.2 ml. of each serum dilution, 0.2 ml. of antigen was added. The tubes were shaken, incubated at 30 C. for three hours and examined by dark ground microscopy using low-power objective and 15 x oculars. Coverslips were not used. The degree of agglutination or "lysis" or both was read as 1+ (with at least 25% of the leptospires agglutinated or "lysed"), 2+ (approximately 50%), 3+ (between 50 to 75%), and 4+ (75 to 100%). The end point was taken as the last dilution showing a 1+ reaction.

Antigens for absorption studies were prepared from five- to seven-day-old cultures grown in Stuart's medium in 500-ml. amounts with the exception of LSU 91, which was grown in Chang's medium. Cultures were killed by the addition of formalin to provide a final concentration of 0.3 per cent. The cultures were centrifuged at 5,000 times gravity in a Servall centrifuge for 25 minutes. The supernatant fluid was discarded, and the leptospires from 250-ml. portions of culture resuspended in 1.0 to 2.0 per cent of the original culture volume with 0.25 per cent formalinized buffered sodium chloride solution. One part of a 1:10 dilution of antiserum was added to 4 parts of the absorbing antigen, mixed thoroughly, incubated in a 50 to 52 C. water bath for two hours and overnight at 28 to 30 C. The cells were re-

TABLE 2—Findings by the Macroscopic Plate-Agglutination Test on Serums from H-Herd

Antigen	Number positive at dilution indicated*					Total tested
	Neg.	10	40	160	640	
L. pomona (S-91)	184	82	19	3	0	288
L. sejroe (M-84)	237	34	12	4	1	288

* This study was performed in June, 1957.

TABLE 3—Agglutinin Absorption Studies on Serum Samples Collected June 5, 1957, from Calves M-592 and M-596**

Calf serum	Antigen *	
	L. sejroe	*L. pomona*
M-592 unabsorbed	20,000	Neg.
absorbed with *L. sejroe*	Neg.	Neg.
absorbed with *L. pomona*	20,000	Neg.
M-596 unabsorbed	20,000	Neg.
absorbed with *L. sejroe*	Neg.	Neg.
absorbed with *L. pomona*	20,000	Neg.
L. sejroe antiserum unabsorbed	20,000	Neg.
absorbed with *L. sejroe*	Neg.	Neg.
absorbed with *L. pomona*	20,000	Neg.

* End point taken as last tube with a 1 + reaction; Neg. = no reaction in a 1:200 dilution.

** These absorptions were performed before the isolate, LSU 91, was identified.

moved by centrifugation and the serum absorbed a second time. To avoid further dilution of the serum, it was added to packed cells. For this second absorption, the serum-cell mixture was incubated two to three hours at 37 C. Absorptions were considered complete when agglutinins were completely removed by the homologous antigen. Microscopic-agglutination tests with the absorbed serums were performed with antigens prepared as described above, which were killed by the addition of formalin to provide a concentration of 0.3 per cent. The absorbed serums were diluted twofold to provide final dilutions of 1:100 to 1:51,200 after the addition of antigen. The tests were incubated in a water bath at 50 C. for two hours, refrigerated for one hour, and read as described previously.

The microscopic-agglutination tests and agglutinin-absorption studies performed on serums from H-herd were conducted by the procedures described above except a different dilution scheme was used. For tests performed on these serums after LSU 91 was identified, a fourfold scheme was used and the end point was taken as the last dilution showing a 4+ reaction (75–100% agglutination).

RESULTS

Preliminary Serologic Studies on H-Herd.—The preliminary serologic findings on H-herd by the macroscopic plate-agglutination test with *L. pomona* and *L. sejroe* antigens are presented (table 2). Reactions against *L. pomona* in dilutions of 1:10 to 1:160 were observed in 104 (36%) of the 288 serum samples and against *L. sejroe* in 50 (17%). One animal showed a titer of 1:640 against *L. sejroe* antigen. Some samples reacted with only one of the antigens and others showed titers for both antigens. Since *L. pomona* and *L. sejroe* are serologically distinct, the presence of a second leptospiral serotype in this herd was suspected.

Two calves, M-592 and M-596, that had macroscopic test titers of 1:160 to *L. sejroe* antigen and no reaction with *L. pomona*, were selected for further serologic studies. In the microscopic-agglutination test with live antigens, serums from both calves were positive in a 1:20,000 dilution to *L. sejroe* and negative to *L. pomona*. In absorption studies, *L. pomona* failed to reduce the titer of the serums to *L. sejroe* but when absorbed with *L. sejroe* no titer could be detected to *L. sejroe* (table 3). With this indication that M-592 and M-596 were or had been infected with a member of the hebdomadis serogroup, an attempt was made to isolate the leptospires.

TABLE 4—Agglutinin Response of Calves 26, 31, and 33 to L. pomona, L. sejroe, and L. hardjo by the Microscopic-Agglutination Test with Live Antigens

Calf 26				Calf 31				Calf 33			
Day*	L.p.	L.s.	L.h.	Day*	L.p.	L.s.	L.h.	Day*	L.p.	L.s.	L.h.
Pre.	Neg.	Neg.	Neg.	Pre.	Neg.	Neg.	Neg.	Pre.	Neg.	Neg.	Neg.
5	Neg.	Neg.	Neg.	5	Neg.	Neg.	Neg.	3	Neg.	Neg.	Neg.
10	Neg.	Neg.	Neg.	7	Neg.	Neg.	Neg.	8	Neg.	Neg.	Neg.
12	Neg.	100	400	9	Neg.	Neg.	Neg.	10	Neg.	Neg.	Neg.
13	Neg.	400	400	11	Neg.	Neg.	Neg.	13	Neg.	Neg.	Neg.
14	Neg.	1,600	1,600	12	Neg.	100	100	14	Neg.	400	400
16	Neg.	1,600	6,400	13	Neg.	400	400	15	Neg.	1,600	1,600
18	Neg.	1,600	1,600	16	Neg.	1,600	1,600	17	Neg.	1,600	1,600
25	Neg.	1,600	1,600	23	Neg.	1,600	1,600	19	Neg.	1,600	6,400
34	Neg.	400	1,600	26	Neg.	400	400	20	Neg.	6,400	6,400
46	Neg.	400	1,600	33	Neg.	100	100	28	Neg.	400	1,600
74	Neg.	400	400	70	Neg.	100	100	65	Neg.	400	1,600
90	Neg.	100	400	103	Neg.	100	100	90	Neg.	400	400
192	Neg.	25	400	144	Neg.	100	100	131	Neg.	400	400

L.p. = *L. pomona*; L.s. = *L. sejroe*; L.h. = *L. hardjo*; Pre. = preinoculation sample; Neg. = no reaction in a 1:25 dilution.

Four plus agglutination (75% or more) reaction used to assess end titer.

* Indicates days after inoculation.

TABLE 5—Cross-Agglutination Studies on Strain LSU 91

No.	Antiserum against	Homologous titer of antiserums	Titer against LSU 91 antigen	LSU 91 antiserums against 1–18 antigens
1	*L. hebdomadis*	12,800	50	3,200
2	*L. medanensis*	12,800	6,400	12,800
3	*L. wolfi*	12,800	12,800	12,800
4	*L. hardjo*	51,200	102,800	25,600
5	*L. sejroe*	6,400	1,600	12,800
6	*L. saxkoebing*	25,600	1,600	3,200
7	*L. wolfi A*	51,200	6,400	25,600
8	*L. kremastos*	6,400	1,600	400
9	*L. borincano*	51,200	25,600	3,200
10	*L. worsfoldi*	102,400	1,600	800
11	*L. hemolyticus*	51,200	1,600	1,600
12	*L. ricardi*	51,200	800	1,600
13	*L. mini A* (szwajizak)	12,800	800	1,600
14	*L. mini AB* (sari)	6,400	100	800
15	*L. jules*	25,600	6,400	800
16	*L. kabura*	102,400	6,400	12,800
17	LT 117	6,400	200	1,600
18	LSU 91	51,200	51,200	51,200

Titer expressed as reciprocal serum dilution; 1 + reaction used to determine end point.

Isolation of Leptospires from Calves.—The first calf (C-26) inoculated with urine from M-592 and M-596 developed agglutinins demonstrable on the twelfth day with *L. sejroe* and *L. hardjo** antigens, but remained negative for *L. pomona* throughout the observation period (*see* table 4).

* The June, 1957, samples from C-26, C-31, and C-33 were tested only with *L. sejroe* and *L. pomona* antigens. All samples were retested in June, 1959, with *L. sejroe*, *L. pomona*, and *L. hardjo* antigens. They were stored at 20 C. during the interval.

Urine from C-26, collected on the 48th and 61st days, infected C-31 and C-33, respectively. It is interesting that leptospires were isolated from the blood of C-31 on the ninth day postinoculation (the only time blood was cultured) and from C-33 on four of the seven days cultures were made. No isolations were obtained from C-33 on the sixth, seventh, and twelfth days. Of further interest is the fact that isolations were obtained only in the modified Stuart's (semisolid) medium (*see* table 1). The leptospiral isolate from C-31 was given the number LSU 91 and will be referred to by that designation in this report.

Identification of Strain LSU 91.—The leptospiral strain LSU 91 was maintained by frequent subculture in the modified Stuart's semisolid medium for several months. After numerous attempts, growth was established in Fletcher's semisolid medium, but it was six months before the strain became adapted to Chang's liquid medium. Earlier trials in four other liquid mediums met with failure. Recently (13 months later) this strain has become adapted to Stuart's liquid medium. To maintain it in liquid medium, subcultures are necessary every five days.

Since serum from calf C-31, from which LSU 91 was isolated, had a high titer against antigen prepared from *L. sejroe*, a relationship of this leptospiral isolate with a member of the hebdomadis serogroup was suspected. The results of cross-agglutination

TABLE 6—Cross Agglutinin-Absorption Studies on Strain LSU 91

Antiserum	Antigen						
	hardjo	*wolfi*	*wolfi* A	*borincano*	*medanensis*	*sejroe*	LSU 91
hardjo (unabsorbed)	12,800	.....	.....	.....	.....	.....	6,400
absorbed with LSU 91	800	.....	.....	.....	.....	.....	—
wolfi (unabsorbed)	.....	6,400	.....	.....	.....	.....	1,600
absorbed with LSU 91	.....	1,600	.....	.....	.....	.....	—
wolfi A (unabsorbed)	.....	.....	6,400	.....	.....	.....	1,600
absorbed with LSU 91	.....	.....	6,400	.....	.....	.....	100
borincano (unabsorbed)	.....	.....	.....	12,800	.....	.....	3,200
absorbed with LSU 91	.....	.....	.....	12,800	.....	.....	—
medanensis (unabsorbed)	.....	.....	.....	.....	3,200	.....	800
absorbed with LSU 91	.....	.....	.....	.....	3,200	.....	—
LSU 91 (unabsorbed)	12,800	6,400	3,200	400	3,200	6,400	12,800
absorbed with *hardjo*	—	.....	.....	.....	.....	.....	400
wolfi	.....	—	—	.....	.....	.....	1,600
wolfi A	.....	.....	—	.....	.....	.....	400
borincano	.....	.....	.....	—	.....	.....	6,400
medanensis	.....	.....	.....	.....	—	.....	6,400
sejroe	.....	.....	.....	.....	.....	—	1,600
LSU 91	.....	.....	.....	.....	.....	.....	—

Titer expressed as reciprocal of serum dilution; Neg. (—) = no reaction in a 1:100 dilution.

reactions of antigen prepared from LSU 91 and antiserums against 17 members of the hebdomadis serogroup are shown (table 5). Cross agglutination-absorption studies were performed against those serotypes with which LSU 91 showed strong agglutination activity (table 6). These studies indicate that LSU 91 is an homologous serotype of *L. hardjo*, according to the rules of nomenclature approved by the Leptospira Subcommittee of the International Committee on Nomenclature. It is interesting to note that according to accepted criteria for identification of leptospiral strains, LSU 91 could be classified also as a subserotype of *Leptospira wolffi* A.

Further Serologic Studies on H-Herd.—Since the serum samples taken from H-herd in June, 1957, were still available after strain LSU 91 was identified as *L. hardjo*, they were tested by the microscopic-agglutination test with live antigens prepared from *L. pomona*, *L. sejroe*, and *L. hardjo*. These findings suggest the presence of both *L. hardjo* and *L. pomona* infection in this herd (table 7). This confirms the previous indications of the macroscopic plate-agglutination tests. The individual serologic patterns representative of the herd findings are presented on 15 animals (table 8). Obviously, the infecting serotype could not be determined definitely by serologic response. In general, titers were slightly higher to *L. hardjo* but in many instances reactions against *L. sejroe* and *L. hardjo* antigens were identical even though occasionally reactions appeared more complete with *L. hardjo*. The few additional positives with *L. sejroe* occurred in the low dilutions.

TABLE 7—Serologic Results on Serums from H-Herd by the Microscopic-Agglutination Test with Live Antigens*

Antigen	Number reacting at dilutions indicated**					Total tested
	Neg.	25	100	400	1,600	
L. pomona, S-91	198	12	49	25	4	288
L. sejroe, Mallersdorf	132	52	74	29	1	288
L. hardjo, Hardjoprajitno	142	25	46	56	19	288

* Serum samples had been stored at —20 C. from June, 1957, until June, 1959, at which time this study was performed.
** Four plus (75% or more) reaction used to assess end titer.

TABLE 8—The Serologic Pattern of Individual Animals by the Microscopic-Agglutination Test with Live Antigen

Animal ident.	Age	Sex	Titer vs. antigen*		
			L. pomona	*L. sejroe*	*L. hardjo* †
134	8 yr.	F	Neg.	Neg.	Neg.
156	8 yr.	F	25	25	25
479	6 yr.	F	25	400	400
11	10 yr.	F	Neg.	100	100
334	6 yr.	F	100	100	400
142	8 yr.	F	400	Neg.	Neg.
139	8 yr.	F	400	25	25
413	4 yr.	F	Neg.	400	1,600
429	4 yr.	F	400	400	1,600
135	8 yr.	F	Neg.	100	1,600
544	18 mo.	F	25	25	1,600
636	8 mo.	F	Neg.	Neg.	Neg.
638	8 mo.	F	Neg.	400	1,600
592**	1 yr.	M	Neg.	400	1,600
596**	1 yr.	M	Neg.	400	1,600

* Four plus (75 % or above) reaction was used to assess end point. ** Urine from 592 and 596 was infective for calf C-26. † Strain Hardjoprajitno.

DISCUSSION

Leptospira hardjo (Hardjoprajitno) was first isolated in 1938 from a human case of leptospirosis in Sumatra.[16] According to Alston and Broom,[1] an animal host of this serotype had not been found previously. Therefore, to the authors' knowledge, the culture reported here represents the first isolation of *L. hardjo* from an animal host.

Antibodies against *L. sejroe* have been found to occur in a high percentage of cattle throughout the United States since 1952,[2,4,7,9,10,15] and they were demonstrated at a significant level in H-herd as well as in the experimental calves. In addition, antibodies against *L. hardjo* in H-herd were comparable or even slightly higher than against *L. sejroe*. These findings, together with the fact that the two serotypes are serologically related, suggest that *L. hardjo* may actually be the infecting serotype in the many other herds that have shown titers to *L. sejroe*.

The presence of antibodies against *L. pomona* in serum from some animals in H-herd and against *L. pomona*, *L. sejroe*, and *L. hardjo* in others suggests past or present infection with *L. pomona* also. Recent studies on dogs[11] experimentally infected first with *L. pomona* and two and one half

months later with *L. canicola* support the fact that animals may shed one serotype after infection with another serotype and at the same time show a higher titer to the second type. This may explain why *L. pomona* was isolated in Florida [3] from a cow having antibodies to *L. sejroe*.

From the limited studies herein reported, the pathogenicity of *L. hardjo* LSU 91 cannot be accurately evaluated. Recent signs of disease cannot be detected in all herds infected with *L. pomona* and even experimentally induced infections may be mild. Thus, even though the experimental *L. hardjo* infections reported here appeared mild, other strains of *L. hardjo* may produce more severe symptoms. It is hoped that studies now in progress will provide more information regarding the degree of disease produced by this serotype. The difficulty involved in isolating this organism on currently available mediums has hampered these efforts considerably. Another herd is presently under study [12] in which experimental evidence indicates some animals have been infected with *L. hardjo* or a closely related member of the hebdomadis serogroup and may be shedding leptospires.

SUMMARY

The isolation and identification of *Leptospira hardjo* from cattle is described. A close antigenic relationship of *L. hardjo* and *Leptospira sejroe* in naturally and experimentally infected animals was shown and indicates that *L. hardjo* may be responsible for the widespread occurrence of antibodies detected with *L. sejroe* antigen in cattle in the United States. The data presented suggest that the same herd may be infected with two leptospiral serotypes. Additional studies to clarify these facts are indicated.

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The Internal Structure and Composition of Siliceous Urinary Calculi of Bovine Origin

RICHARD F. KEELER, Ph.D.

Bozeman, Montana

IN THIS laboratory, work in the area of urolithiasis has been directed in part at an attempt to establish the chemical nature [1,2] and mechanism of formation of siliceous calculi of bovine origin. The experiments reported here were designed to characterize further the nature of the constituents of siliceous calculi and their relationship to internal structure by the use of staining techniques, by microautoradiography, and by chromatographic analysis.

METHODS

Sectioning of Stones.—Stone sections were prepared for staining by cutting the stones in half with a 0.5-mm. thick emery wheel mounted in a hand grinder. The halved stone was then mounted flat side up on a glass slide using Du Pont Duco cement. The flat portion of the stone was ground and polished using emery cloth, powdered corundum, and jewelers' rouge.

Staining methods.—Prior to staining, the sections were fixed in 20 per cent formalin in absolute ethyl alcohol for all stains except Alcian blue. For the latter, Susa [14] fluid was used. For staining with mucicarmine and toluidine blue, modifications of the methods of Hammerman [8] for chromatography were used. The method of Laskey as described by Lillie [15] was used for mucihematein staining. The periodic acid-Schiff (PAS) technique was also from Lillie [16] as was that for Sudan black B and Hale's iron.[17] The method of Steedman [20] was used for staining with Alcian blue. The trichrome staining technique was the one described by Gomori.[7] At least ten to 15 sectioned stones were stained by each method. Each stone was from a different animal.

After the sections were stained, washed, and dried, a toluene-damar protective coating was poured over each specimen.

Color photographs of the sections were prepared under incident light at a magnification of x 30.

Microautoradiography.—Both whole and sectioned stones were allowed to stand in carrier-free solutions of P^{32} and Ca^{45}. Ten sectioned stones and four whole stones were placed in each solution. The P^{32} system contained 1.34 mc. in 200 ml. of distilled water, and the Ca^{45} system contained 0.0027 μc. of Ca^{45} in 200 ml. of distilled water when the experiment began. The stones and sections were allowed to stand in these solutions for six weeks at 32 C. The samples were shaken twice a day. After this period, the stones were repeatedly washed in distilled water using a camel's-hair brush to remove adhering radioactivity. Presumably this removed most adhering microorganisms which may have grown during contact with the radioactivity, which may have incorporated activity, and which may have adhered to the stone surface. The whole stones were then cut in half and the halves prepared as described previously. These sections, as well as those from stones placed in the solutions as sections, were coated with Saran* in methylethyl ketone as described by Plumb [19] to minimize chemical fogging of the nuclear track plate. The microautoradiograms were prepared [19] using Kodak nuclear track plates type NTB with a 10-μ emulsion thickness. Exposure time was three to six days for stones which incorporated P^{32} and 30 days for those which incorporated Ca^{45}. The plates were developed for four minutes in Kodak D 19, washed ten minutes in running water, fixed in acid fixer for 20 minutes, and washed for one hour in running water (68 F. held throughout). Enlarged negatives (x 4) were made from the nuclear track plates. The contact prints shown (fig. 19–22) were made from negatives prepared in this way.

Chromatographic Methods.—Stone hydrolysates were prepared from ground stone material using acid-regenerated Permutit Q** after the methods of Glegg and Eidinger.[6] Chromatography of the hydrolysates after vacuum drying and resuspension was by triple ascension in *n*-propanol: ethyl acetate: water (7:1:2), and in *n*-butanol: acetic acid: water (4:1:5). The upper layer of the latter was used as the moving phase and the lower layer as equilibrant. The chromatograms were then dipped in benzidine-acetate [1] to disclose the location and relative concentrations of the sugars. Standard sugars were chromatographed on the same paper as the hydrolysates.

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** Produced by the Permutit Co., New York, N.Y.

RESULTS

Internal Structure.—The most common urinary stones found in clinical cases of urolithiasis in range steers in the Montana area were 4 to 7 mm. in diameter and varied from a rough to a fairly smooth outer surface (fig. 1). The internal structure varied between two extremes, a laminated type with no embedded stones, and an essentially nonlaminated type containing small embedded stones of varying sizes. There was little correlation between these two extremes of internal structure and the texture of the outer surface. Stained sections from examples of these two extremes are shown (fig. 2, 3). Many stones were of an intermediate structural type containing both laminations and small embedded stones. Frequently the small embedded stones themselves were laminar, although some were found to be entirely amorphous. The latter were sometimes inorganic, sometimes organic in composition.

Further lamination detail in the predominantly laminar stones is shown (fig. 4–7). Even though these two stones were predominantly laminar, some small embedded stones were found. In some stones (fig. 4), the small embedded stones seemed to be rather randomly distributed. In others (fig. 6), some were randomly distributed and others made up the central "clumps" around which the main stone formed. It is evident from the enlarged areas (fig. 5, 7) that the laminations were not discrete, uniform rings but rather quite variable, although occasionally stones were seen showing greater uniformity in laminar structure than those shown (fig. 4–7).

Specific Staining.—Many available stains have a degree of specificity for certain compounds. There are a few stains with some specificity for mucoproteins and mucopolysaccharides. These stains have been used on sections of siliceous urinary stones of bovine origin in an effort to further characterize the mucoprotein matrices of these stones.

Stone sections stained with mucicarmine, a general stain for mucoprotein, gave strongly positive results. An intense reddish pink was evident in laminated stones (fig. 8), as well as in the amorphous material of the nonlaminated stones (fig. 9).

Mucihematein, another stain which is fairly specific for mucoproteins, yielded the positive violet color with all sections tried (fig. 10).

Toluidine blue shows an orthochromatic (blue) color on many types of mucoproteins, including those with hexuronic-acid mucopolysaccharide moieties, but shows a distinct metachromatic (purple to red) color with sulfonated acidic mucopolysaccharide moieties. In sections stained by this method, metachromasia was not seen, suggesting the absence of sulfonated acidic mucopolysaccharides. However, strong orthochromatic color was evident (fig. 11–13). This further suggested the presence of mucoprotein in the laminations, in the amorphous areas, and in the small stones. The central stone (fig. 11) was largely inorganic as evidenced by the lack of staining. Two central stones which were largely mucoprotein as judged by their deep orthochromatic staining are shown (fig. 13).

All sections stained with the PAS tech-

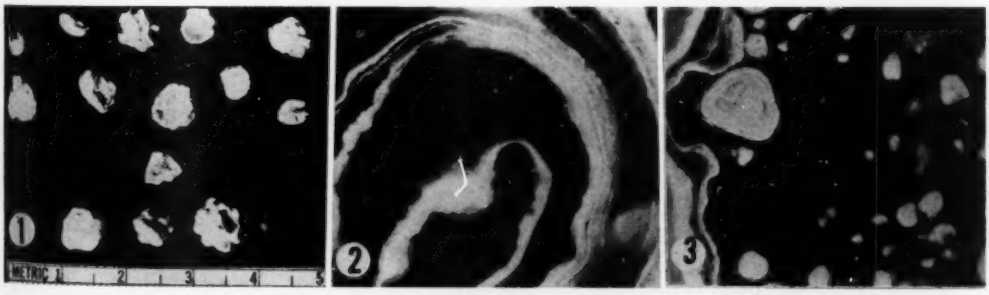

Fig. 1—The gross appearance and size of typical siliceous urinary stones of bovine origin are shown. x 25.

Fig. 2, 3—Sections from siliceous stones stained with trichrome which show the extremes of internal structure from the generally laminar to that type composed of many small stones embedded in a mucoprotein matrix. x 25.

XUM

nique, which is specific for mucoproteins in general, gave the positive red-purple color (fig. 14–16). Lipids and glycogen may also give positive results. However, it is unlikely that the positive results were due to lipids since Sudan black B, a stain specific for lipids, failed to yield positive results on any section tried. Furthermore, it is unlikely that the positive results obtained by the PAS method were due to glycogen, since there was such a small amount of glucose present in the stones (table 1).

Steedman devised a staining method using Alcian blue, which is specific for mucoitin and chondroitin sulfate-type mucopolysaccharides. Sections stained by this method were all negative, again suggesting the absence of sulfonated acidic mucopolysaccharide moieties in these stones.

Representative sections stained by Hale's iron method for mucopolysaccharides are shown (fig. 17, 18). All sections stained by

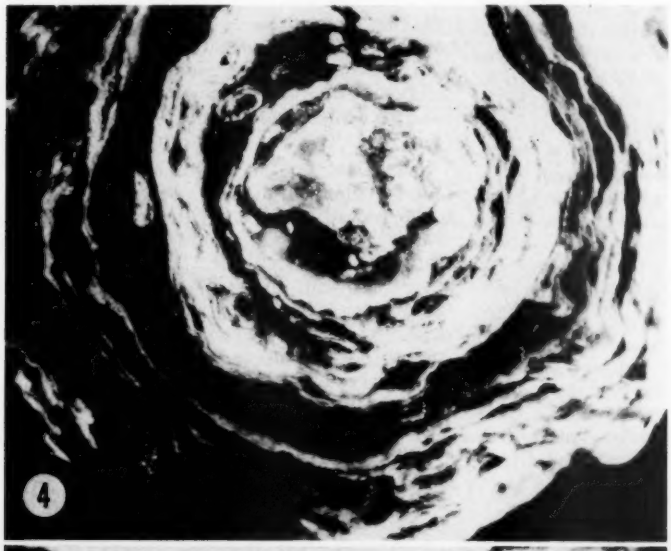

Fig. 4—A section from a siliceous urinary stone of bovine origin stained with mucicarmine is shown. x 30.

Fig. 5—Higher magnification of section shown in figure 4, showing greater detail of the internal laminations. x 85.

this method gave the positive deep-blue color.

Microautoradiography.—It would be of interest to know the relative degree of permeability of stone laminations to inorganic ions which comprise the stone. This information would be an aid in elucidating the mechanism [2,3,5] of stone formation. Knowledge of the degree of permeability would also be required to test the feasibility of using radioactive isotope-incorporation techniques for studying the rate of formation of artificial stones.[11] This might be accomplished by the *in vivo* incorporation of a radioactive isotope into discrete stone laminations during their formation. Microautoradiography of sections of such stones could give information on the amount of deposit as a function of the environmental conditions.

To test the permeability of the laminar siliceous stones, and to assess the exchange

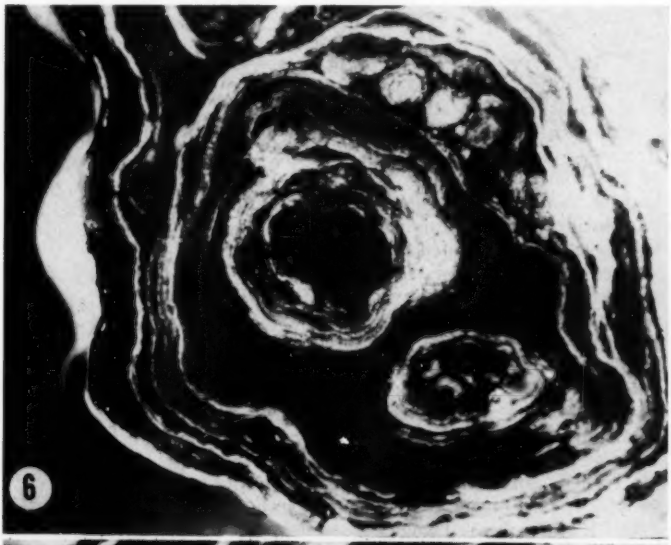

Fig. 6—A section from a siliceous urinary stone of bovine origin stained by Hale's iron method is shown. x 30.

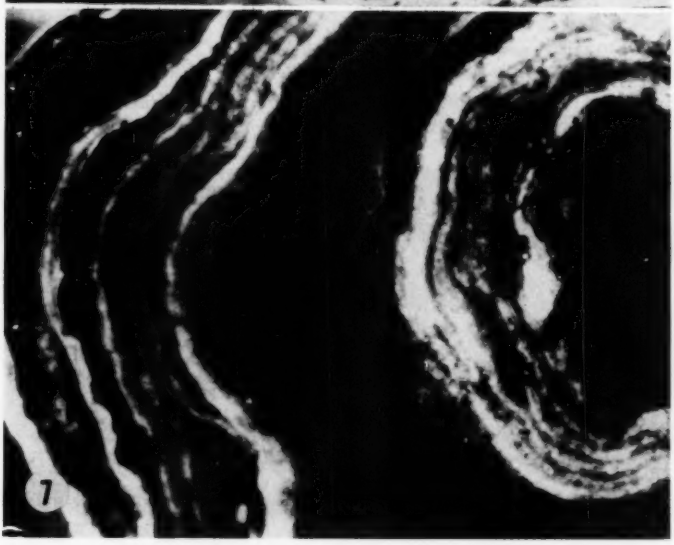

Fig. 7—Higher magnification of section shown in figure 6, showing greater detail of the internal laminations. x 75.

TABLE 1—The Relative Intensities of Spots on a Sugar Chromatogram of Permutit Q Hydrolyzed Siliceous Bovine Stone Material

Sugar present	Relative intensity of spot
Galactose	++++
Mannose	+++
Fucose	++
Rhamnose	++
Glucose	+

of these elements with their respective counterparts in the stone,[4] P^{32} and Ca^{45} were used. These two isotopes were used because no long-lived isotope of silicon is as yet available. The results shown (fig. 19–22) are representative of experiments where P^{32} was used. Microautoradiograms of flat sections of stones placed in P^{32} are shown (fig. 19, 20). It is evident that appreciable exchange, or adsorption, or both, of P^{32} took place on the flat surface. The lack of much laminar detail suggests that adsorption may predominate, even though the sections were repeatedly washed as described in the methods section. Two whole stones which were placed in the P^{32} solution and then sectioned for microautoradiography are shown (fig. 21). It was evident that most of the P^{32} was present on the outside surface of the stones. The stones did, however, show some permeability since a small amount of P^{32} apparently penetrated some of the outer mucoprotein laminations. No other whole stones checked showed greater permeability than the one shown (fig. 21, right side). Two other stones placed in P^{32} and four stones placed in Ca^{45} showed only a trace of penetration of the radioactive isotopes across the laminations.

Penetration (apparent exchange) of the isotopes did occur in flat sections. A microautoradiogram is shown of a stone section containing P^{32} prepared after an additional 1-mm. thick section of stone material had been removed from the flat surface and discarded (fig. 22). This essentially removed surface-adsorbed radioactivity. Some lamination detail can be seen in this microautoradiogram as one would expect if exchange of P^{32} with the phosphorus of the stone had occurred. Notice in this case that the P^{32} did not have to cross the mucoprotein laminations but could penetrate between them.

Experiments with Ca^{45} showed similar results to those with P^{32}, except that less incorporation occurred. This, coupled with the low energy of the Ca^{45} isotope (0.025 MEV β^-) and the lower level of radioactivity used, resulted in less intense microautoradiograms even though the exposure of the plates was five to six times longer. The microautoradiograms with Ca^{45} were not considered suitable for photographic reproduction.

Sugar Composition by Paper Chromatography.—It was reported earlier[12] that chromatographic analysis of acid-hydrolyzed stone material indicated the presence of glucose, galactose, mannose, and rhamnose. With such acid hydrolysis, it was difficult to obtain completely salt-free samples and the chromatograms were somewhat unreliable due to salt effect. Recently, chromatography has been tried with hydrolysates obtained using acid-regenerated Permutit Q.[6] The results have been extremely good and have allowed both qualitative and semiquantitative estimation of the sugars present.

Results obtained from a hydrolysate of a pool of 14 typical siliceous stones run

Fig. 8, 9—Sections of siliceous urinary stones of bovine origin that were stained with mucicarmine. x 25.

Fig. 10—Section of a siliceous urinary stone of bovine origin that was stained with muci-hematein x 25.

chromatographically in *n*-propanol:ethyl acetate:water (7:1:2) and stained with benzidine-acetate are shown (table 1). Hydrolysis for 16, 24, or 48 hours yielded identical results, even though it has been reported [6] that hydrolysis of 48 hours' duration or longer results in some loss of both fucose and rhamnose. Similar results were obtained with two other pools, as well as with 14 hydrolysates from individual stones. It is interesting that the sugar composition of the individual stones was uniform both qualitatively and quantitatively. A minor exception was that with six of the stones an additional spot was evident which proved to be xylose. The intensity of this spot varied from one stone to another. In one case, it had a value of about two plus (++), and in the five other cases its value was one plus (+) or less. The spot was not evident in the other 11 hydrolysates. In one hydrolysate of a single stone, glucose was found to have a value of four plus (++++) rather than a one plus (+) as in all other cases.

Chromatography in another solvent, *n*-butanol:acetic acid:water (4:1:5), for further comparison of R_f values of the sugars resulted in verification of the results obtained with the *n*-propanol:ethyl acetate:water system.

Fig. 11–13—Sections from siliceous urinary stones of bovine origin that were stained with toluidine blue. x 25.

Discussion

Since stone sections did not stain with Alcian-blue, and since there was no metachromasia evident with toluidine blue, one might conclude that the mucoprotein of stone matrices from siliceous stones of bovine origin contains no sulfonated acidic mucopolysaccharide moieties. Dang [4] was unable to demonstrate the presence of any significant amounts of sulfur in siliceous stones by chemical analysis, and Keeler and Swingle [12] were unable to detect hexuronic acid in stone material. These findings, along with the high content of neutral sugars and the strong PAS reaction in stone sections reported here, suggest that stone matrix is of the neutral mucoprotein type.

The uniformity in carbohydrate composition (except for traces of xylose in some

Fig. 14–16—Sections of siliceous urinary stones of bovine origin that were stained by the periodic acid-Schiff method. x 25.

stones) suggests constancy in the mucopolysaccharide moiety from stone to stone. This is apparently so, even though the amino acid concentrations do vary among stones.[12] One might propose on this basis that the mucoprotein of stone matrix is rather uniform but that other protein(s) is incorporated to a varying degree. In contrast to the lack of uniformity in amino acid concentrations from stone to stone reported by Keeler and Swingle,[12] King and Boyce [13] have reported a greater degree of uniformity in stones taken from human beings.

A theory that a partially or completely preformed mucoprotein matrix [2,3] is required upon which inorganic material can be deposited during the stone-forming process would seem to require that diffusion or exchange of the inorganic materials takes place rather readily into and out of the stone. Boyce and King [3] recognized this, and stated that diffusion of ions into large, intact calculi can be readily demonstrated *in vitro* by immersion in dilute solutions of toluidine blue. However, as they further stated, clinical observations suggest that the calculi rarely decrystallize *in vivo*, perhaps because of some selectivity imposed on ion migration. It would seem, on the basis of the results reported here for Ca^{45} and P^{32}, that very little diffusion and exchange of such ions can occur across the mucoprotein laminations of siliceous bovine stones. This is reminiscent of the results on the incorporation of P^{32} into bone material reported by McCann and Fath.[18] They suggested that the slow P^{32} exchange into whole bones was due to the protein matrix slowing the diffusion or exchange process, since ashed bone material rapidly exchanged P^{32}.

Aside from theoretical considerations on the mechanism of the stone-forming process, the radioactive isotope-incorporation technique offers an interesting tool which may be useful in studying the rate of formation of artificial stones [11] as a function of environmental conditions. Since diffusion and exchange of isotope (P^{32} and Ca^{45}) does not readily occur across the mucoprotein laminations, one might expect that if an isotope were incorporated into a certain area or certain lamination during formation, most of it would be retained in that lamination. Thus, assuming stone formation occurs layer upon layer rather than by diffusion into a preformed matrix, one might expect that upon short-term administration of an isotope, say P^{32}, to an animal forming an experimental calculus,[11], incorporation into a single or limited number of laminations would occur. After cessation of P^{32} administration, subsequently formed laminations should be relatively free of the isotope, provided exchange from other parts of the body did not cause significant urine levels of P^{32}. Exposure periods to various environmental conditions could then be alternated with periods of isotope administration. The amount of deposited material between laminations containing the isotope would serve as a measure of the effect of the environmental schedule. However, if stone formation occurs by diffusion of inorganic constituents into a large preformed matrix, the results of such an experiment would probably be more difficult to interpret,

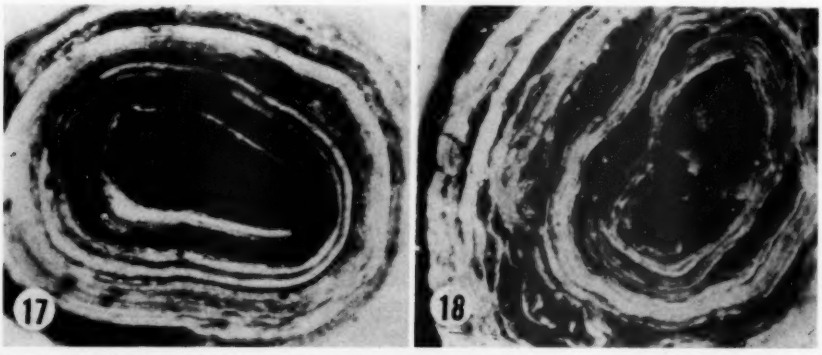

Fig. 17, 18—Sections of siliceous urinary stones of bovine origin that were stained by Hale's iron method. x 30.
All stones shown in figures 19, 20, 21, and 22 were essentially of the laminar type. x 4.

since the radioactivity might be rather uniformly distributed throughout the stone.

The following factors must be considered in any theory of mechanism of formation of siliceous bovine stones. The silica of these stones is not crystalline, but is mainly amorphous ($SiO_2 \cdot nH_2O$).[5] Protein and silicic acid are known to undergo coprecipitation under certain conditions.[9] Silicic acid alone will also polymerize or gel under certain conditions.[10] In contrast to stones from human beings, where no calculi with completely "amorphous" matrices containing no "fibrous" laminations are seen,[3] bovine stones may at times be composed almost exclusively of amorphous matrices with only a few laminations at the outer surfaces (fig. 3, 9, 11, 13, 14). As reported here, there is only a slight, or perhaps complete lack of diffusion and exchange of ions (P^{32} and Ca^{45}) across the mucoprotein laminations. There is no evidence to suggest that the amorphous matrix areas seen in these stained stone sections are necessarily exclusively organic, even though they stained as mucoproteins. It may be that they are silicon-mucoprotein coprecipitates. Laminations may simply be layers of varying proportion of silicon to mucoprotein, the ratio of which depends upon ambient urine concentrations.

SUMMARY

The use of staining techniques with mucicarmine, toluidine blue, mucihematein, Alcian blue, Sudan black, trichrome, Hale's iron, and the periodic acid-Schiff (PAS) methods revealed considerable detail of the internal structure of siliceous calculi of bovine origin. The results have shown that the internal structure of these stones varies between extremes from the laminated types to the nonlaminated types which may contain smaller stones of varying sizes embedded in an amorphous mucoprotein matrix. The results reported here, based on the specificities of the stains along with earlier published observations,[12] suggest

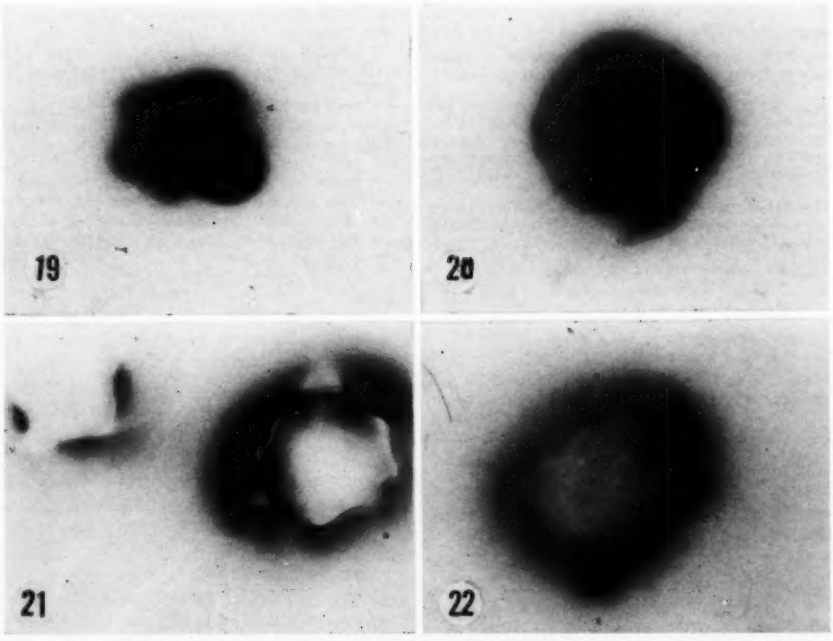

Fig. 19, 20—Microautoradiograms of sections of siliceous urinary stones of bovine origin that were placed in solutions of P^{32} as sections.

Fig. 21—Sections of stones placed in P^{32} as whole stones and then sectioned for microautoradiography are shown.

Fig. 22—A microautoradiogram of a sectioned stone placed in P^{32}, but prepared after 1 mm of stone material had been removed from the flat surface.

that the mucoprotein matrices of both the laminar and amorphous stones are of the nonacidic type.

Microautoradiograms of whole, laminar stones placed in solutions of P^{32} or Ca^{45} showed only limited diffusion or exchange of these isotopes into the whole stone; *i.e.*, through the laminations. Microautoradiograms of sectioned stone material placed in solutions of these isotopes showed a rather extensive diffusion or exchange, apparently parallel to the laminations. The implication of these results in the mechanism of stone formation and the possible use of the techniques as an experimental tool for studying the rate of formation of stones is discussed.

By chromatographic analysis, the mucopolysaccharide portion of the mucoprotein matrix has been shown to contain the following sugars at the relative concentrations listed: galactose (++++), mannose (+++), fucose (++), rhamnose (++), and glucose (+). In some stones, there was a trace of xylose at relative concentrations of (+) or less.

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Prevention of White Muscle Disease in Lambs by Oral and Subcutaneous Administration of Selenium

KENNETH L. KUTTLER, D.V.M., M.S., and DONALD W. MARBLE, D.V.M.

Reno, Nevada

RECENT DEVELOPMENTS in white muscle disease (WMD) research have provided needed methods for studying ways of preventing this condition. Legume rations have long been associated with WMD, but in our area it has been only recently that alfalfa hay, from ranches with a history of this condition, has been utilized to produce experimental WMD in lambs. Whiting *et al.*[12] reported success in reproducing WMD in lambs by feeding poor quality hay and cull beans to the ewes during gestation. Oldfield *et al.*[9] more recently found that legumes from certain farms and areas appeared to be responsible for WMD when fed experimentally to sheep during gestation

A condition similar to WMD can be consistently induced by feeding unsaturated fats along with a ration deficient in vitamin E.[1,3,11] Blincoe and Dye[2] reported a marked increase in serum glutamic oxalacetic transaminase (SGO-T) associated with WMD. Other workers[3,10] have demonstrated the reliability of SGO-T levels in indicating early and subclinical cases of WMD, as well as aiding in the diagnosis of clinical cases of WMD. With the SGO-T test and the ability to produce WMD in lambs from ewes fed hay from ranches with histories of WMD, efforts to develop effective preventive measures have been made easier.

Recent research at this station has indicated the effectiveness of a vitamin E supplement in preventing WMD when creep-fed to lambs.[4] Muth *et al.*[8] found that by adding selenium at the rate of 0.1 part per million (p.p.m.) to the ration of ewes during gestation, WMD was prevented in the lambs. The procedure of adding selenium to the rations is not always convenient, and also may constitute a hazard due to its toxicity.

The purpose of this study was to determine the effectiveness of injecting a relatively insoluble selenium salt which would release selenium slowly over a long period of time. An effort was also made to repeat the Oregon work[8] by feeding selenium orally to ewes during gestation. In addition, selenium was fed to lambs to determine its prophylactic effects against a dystrophogenic ration containing unsaturated fats calculated to induce a typical muscular dystrophy.

MATERIALS AND METHODS

Preparation of Selenium Salt.—Barium selenate ($BaSeO_4$), selected as a suitable insoluble salt, was prepared by adding 3 Gm. of barium chloride dissolved in 20 ml. of water to 20 ml. of selenic acid (H_2SeO_4). A white precipitate, $BaSeO_4$, was filtered off, washed with water to remove possible soluble residues of selenium, and dried in a hot-air oven. This powdered barium selenate was added to a mixture containing 2 per cent beeswax and 98 per cent peanut oil so as to contain 0.2 Gm. of barium selenate per milliliter. This was mixed in a tissue homogenizer to form an even suspension in the oil, then bottled and autoclaved prior to injection.

Experiment 1, Ewes.—In experiment 1, six groups of ewes (table 1) were used to test the effectiveness of selenium in preventing WMD in offspring when fed hay from ranches known to have histories of WMD. This hay was fed during the last three or four months of gestation and about two months after parturition. Groups 1, 2, 3, and 4 were given dystrophogenic hay from one source and groups 5 and 6 from a second source.

The 5 ewes in group 1 (2 Rambouillets, 3 Hampshires) were 3 to 6 years old. These ewes were each given a subcutaneous injection of the oil mixture at the rate of 0.25 ml. (0.05 Gm. $BaSeO_4$) per 100 lb. of body weight three to ten weeks prior to parturition.

The 6 ewes in group 2 (4 Hampshires, 2 Columbias) were 3 to 6 years old. They were each given a subcutaneous injection of the oil mixture at the rate of 0.50 ml. (0.10 Gm. $BaSeO_4$) per 100 lb. of body weight four to eight weeks prior to parturition.

The 6 ewes in group 3 (3 Rambouillets, 2 Hampshires, 1 Columbia) were 2 to 6 years old. Each was fed sodium selenite (Na_2SeO_3) mixed with 0.20 lb. of rolled barley to provide the equivalent of 0.1 p.p.m. of selenium, based on their total daily ration. The selenium supplement was added to the ration approximately two months prior to

From the University of Nevada Agricultural Experiment Station, Reno, where Dr. Kuttler is associate veterinarian and Dr. Marble is assistant veterinarian.

parturition, and was continued for about two months after parturition.

In group 4, the 9 ewes (4 Rambouillets, 4 Hampshires, 1 Columbia) were 2 to 7 years old. They were given only dystrophogenic hay for the last three or four months of gestation, and approximately two months after parturition.

Group 5 consisted of 16 ewes of undetermined ages and mixed breeding (principally Suffolk stock). These ewes were each given a subcutaneous injection of the oil mixture at the rate of 0.50 ml. (0.10 Gm. $BaSeO_4$) per 100 lb. of body weight for approximately six weeks prior to parturition.

Group 6 consisted of 15 ewes of undetermined ages and mixed breeding (principally Suffolk stock) which served as untreated controls for ewes in group 5.

Experiment 2, Lambs.— In experiment 2, 11 lambs (table 2) of various breeds from a ranch with no history of WMD were weaned at about 5 days of age and fed a vitamin E-deficient ration. Their dams had been fed high-quality alfalfa hay from a ranch with no WMD. When the lambs averaged 32 days of age, they were given a cod liver oil-skim milk ration[3, 4] to produce muscular dystrophy.

The SGO-T test was used to identify WMD. Clinical observation and necropsy findings[6, 7] confirmed the diagnosis. The SGO-T tests, done in the manner previously described,[4] are recorded as *sigma* Frankel units per milliliter (s.f.u./ml.). Levels of over 200 s.f.u./ml. were considered indicative of WMD.

RESULTS

The results (table 1) confirm previous findings of the effectiveness of oral selenium and demonstrate the usefulness of injectable selenium in preventing WMD. No local or systemic ill effects were noticed in any of the animals given injections. In experiment 1, a total of 102 observations were made on 34 lambs from ewes that were given injections of barium selenate. Only 1 lamb (group 5), about 10 days old, showed a transaminase level over 200 s.f.u./ml. There was no other evidence of WMD during the observation period. There appeared to be no difference in the degree of protection between the 0.05 Gm./100 lb. and the 0.10 Gm./100 lb. dosage rate of barium selenate. There was no evidence of WMD in 30 observations on 10 lambs from ewes given selenium orally. With only one exception, lambs from ewes given selenium showed consistently normal average SGO-T levels with low standard deviations, indicating the lack of abnormal variation. The exception was 1 lamb in group 5 which had an SGO-T level of 211 s.f.u./ml. This increased the standard deviation to 51, even though the average of 73 s.f.u./ml. for the group was still well within the normal range.

TABLE 1—The Effects of Selenium on the Incidence of White Muscle Disease in Lambs from Dams Fed Dystrophogenic Hay

Group	No. of ewes	Treatment of ewes	Average SGO-T of lambs expressed as *sigma* Frankel units per ml.					
			No. of lambs	10 days of age	No. over 200	24 days of age	No. over 200	38 days of age	No. over 200
1	5	0.05 Gm. $BaSeO_4$/100 lb. body wt., subcutaneously	7	65 ±29	0	54 ±12	0	63 ±30	0
2	6	0.1 Gm. $BaSeO_4$/100 lb. body wt., subcutaneously	7	60 ±15	0	46 ±8	0	51 ±16	0
3	6	0.1 p.p.m. Na_2SeO_3, orally	10	56 ±11	0	59 ±21	0	65 ±22	0
4	9	Untreated controls	14	173 ±233	3	908 ±1,907	5	591 ±914	8
5	16	0.1 Gm. $BaSeO_4$/100 lb. body wt., subcutaneously	20	73 ±51	1	50 ±21	0	59 ±37	0
6	15	Untreated controls	20	68 ±28	0	234 ±523	3	196 ±355	4

Four of these lambs (2 Hampshires, 1 Rambouillet, 1 Columbia) were designated group A and were given 200 units of vitamin E* daily in the cod liver oil-skim milk ration.

The 4 lambs in group B (2 Hampshires, 2 Rambouillets) were fed 1 p.p.m. of selenium, as sodium selenite, in the milk.

The 3 lambs in group C (2 Hampshires, 1 Rambouillet) were given only the cod liver oil-skim milk ration. These experiments were terminated 45 days after the start of feeding the dystrophogenic rations.

* Vitamin E, supplied by Distillation Products Industries, Rochester, N.Y., was the water dispersible type F-50 containing 50,000 units of vitamin E per gram.

TABLE 2—The Effects of Oral Selenium on Artificially Induced White Muscle Disease in Lambs

Group	Treatment	No. of lambs	Days after start of experiment (av. SGO-T values)						
			3	10	17	24	31	38	45
A	Dystrophogenic ration * plus 200 units vitamin E daily	4	194	138	88	38	107	75**	83
B	Dystrophogenic ration * plus 1 p.p.m. selenium	4	363	98†	179	523	1,013	1,868	1,157
C	Dystrophogenic ration * only	3	146	1,550†	880	1,760	1,515	660	771

* Cod liver oil-skim milk ration. ** One lamb of this group died of a hemorrhagic enteritis; no evidence of white muscle disease was seen. † One lamb of this group died showing typical gross lesions of white muscle disease.

Several of the 34 lambs from untreated controls (groups 4 and 6) showed evidence of WMD, characterized by increases of SGO-T levels, clinical signs, and gross pathologic changes similar to those previously described.[5,6,7] One lamb from each group was necropsied. The results (table 1) show a higher incidence of WMD in group 4 offspring, with 3 lambs (21%) showing SGO-T levels of over 200 s.f.u./ml. at 10 days of age; 5 (36%) at 24 days of age; and 8 (57%) at 38 days of age. No 10-day-old lambs in group 6 showed evidence of WMD; however, SGO-T levels of over 200 s.f.u./ml. developed in 3 (15%) at 24 days of age and in 4 (20%) at 38 days of age.

Of the 11 lambs in experiment 2 (table 2) fed the dystrophogenic ration of cod liver oil and skim milk, only those lambs given vitamin E supplement did not develop clinical evidence of WMD. The addition of selenium to their dystrophogenic ration, at 10 times the amount successful in ewes, was not effective in preventing WMD in 4 lambs.

Two lambs in group A (supplemental vitamin E) developed SGO-T levels of over 200 s.f.u./ml. on treatment days 3 and 10, respectively (table 2). Thereafter, SGO-T levels in all lambs in this group remained within the normal range of less than 200 s.f.u./ml. One death occurred at day 38 in group A, but necropsy failed to reveal any evidence of WMD.

One lamb in group B (supplemental selenium) died on day 6 after showing typical signs of WMD. This was confirmed by gross lesions at necropsy. On day 24, all the remaining animals of this group showed SGO-T levels in excess of 200 s.f.u./ml.

On day 16, 1 lamb in group C (untreated controls) died after showing typical signs of WMD. The diagnosis of WMD was confirmed by gross lesions at necropsy. Both of the other lambs developed SGO-T levels of over 200 s.f.u./ml. on day 17, followed by clinical signs. In order to minimize needless losses, treatment with vitamin E and removal of cod liver oil from the ration was instituted in 1 lamb of group C at day 31 and in all surviving lambs after day 45.

Discussion

The incidence and intensity of WMD in the control animals was not as great as it has been in the past, which makes it important to repeat these experiments when the disease is more intense. However, there appears to be adequate indication of the usefulness of selenium in preventing the naturally occurring form of the disease. In many localities, a single injection of selenium would be preferable to feeding it daily. Such a procedure may be feasible.

In preliminary trials, sodium selenite injected subcutaneously resulted in acute toxicity. Six out of six 70- to 80-lb. lambs given sodium selenite at levels of 0.075 Gm./100 lb. of body weight and over died within 24 hours after injection without showing marked signs or lesions. The animals were depressed and hard to move two to three hours prior to death. Necropsy findings did not show any gross pathologic changes consistent in all lambs. The sodium selenite has been injected safely at a level of 0.01 Gm./100 lb. of body weight. Preliminary trials with barium selenate indicate no toxicity when injected in amounts up to 0.80 Gm./100 lb. of body weight. This is eight times more than the largest dose used in these experiments. Such large injections tend to produce sterile abscesses, but no systemic changes have been noticed. Studies on selenium levels in serum, while incomplete, showed measurable amounts three months after injection. Injection at the rate of 0.05 Gm./100 lb. of body weight appeared

to be as effective as 0.10 Gm./100 lb., indicating the possibility that even smaller amounts may be sufficient for protection.

The failure of selenium to protect lambs on dystrophogenic rations of cod liver oil and skim milk indicates differences in etiologic factors between the artificially induced and the naturally occurring conditions, even though the final pathologic processes are similar.

SUMMARY

When 27 ewes were given subcutaneous injections of barium selenate at levels of 0.05 Gm. and 0.10 Gm./100 lb. of body weight, their lambs were protected from evidence of white muscle disease (WMD), even though the ewes were getting dystrophogenic feed. Selenium, as sodium selenite, when fed at the rate of 0.1 p.p.m. to 6 ewes, beginning about two months before parturition and continuing two months after parturition, was adequate to prevent WMD in their lambs.

A cod liver oil-skim milk ration containing selenium at the rate of 1.0 p.p.m. (as sodium selenite) did not prevent the development of high serum glutamic oxalacetic transaminase levels, clinical signs, and lesions typical of WMD in lambs.

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Effects of Certain Antibiotics in the Treatment of Vibriosis in Sheep

C. V. HULET, Ph.D.; S. K. ERCANBRACK, Ph.D.;
D. A. PRICE, Ph.D.; R. D. HUMPHREY, M.S.;
F. W. FRANK, D.V.M.; W. A. MEINERSHAGEN, B.S.

Dubois and Caldwell, Idaho

THE GENERAL CHARACTERISTICS of vibrionic abortion of sheep and progress toward achieving a control for this disease have been summarized by Frank *et al.*[1] Ryff and Breen[3] found no beneficial effects from single doses of tetracycline HCl, oxytetracycline, and streptomycin in field trials with sheep affected with *Vibrio fetus,* although normal sheep given comparable amounts of the antibiotics developed serum levels which would inhibit the organism. A possible explanation for this discrepancy may be that the blood levels attained in treated ewes were too transitory to eliminate infection already present or to prevent subsequent infection before immunity could be established.

Frank *et al.*[1] reported that chlortetracycline (80 or 400 mg. per ewe fed daily from 11 days prior to exposure until termination of pregnancy) was relatively effective as a preventive of vibrionic abortion. These same workers[2] recently have found that two consecutive daily injections of 4 cc. of a suspension containing 800,000 units procaine penicillin G and 1 Gm. of dihydrostreptomycin sulfate accompanied by chlortetracycline feeding (½ lb. of grain pellets per ewe daily, containing 80 mg. chlortetracycline) was an effective therapeutic treatment in experimental groups of ewes.

The following experiment was designed to determine the effectiveness of two injections on consecutive days of penicillin-streptomycin* alone and in combination with chlortetracycline* (in grain pellets fed daily), and chlortetracycline alone, in reducing losses due to *V. fetus* in an established epizootic of vibriosis.

MATERIALS AND METHODS

Vibriosis occurred at the U.S. Sheep Experiment Station in 1958 in both a band of Rambouillet ewes and a band of Targhee and Columbia ewes.

Approximately ten days before the first live lamb was expected in the Rambouillet ewe band (1,444 head), a few ewes began to abort on the winter feed lot. At that time these ewes were being fed alfalfa hay on the ground and were being watered from deep wells. The ewes were trailed to station headquarters on March 24 to 26 for lambing. Several aborted and 1 or 2 died on the trail. Nine ewes aborted during the first night after arrival at the station on March 26, and several ewes aborted each succeeding day. A tentative diagnosis of vibriosis made on March 28 was based on the demonstration of typical vibrios in uterine smears from ewes which had recently aborted. The preliminary identification was later verified by the isolation and identification of the organism from several aborted fetuses.

Sheep in the Targhee-Columbia band (1,479 head) began aborting on a separate feed lot (5 miles from the Rambouillet band) only a day or two later than the Rambouillet band and about three weeks before the first live normal lambs were born. These ewes had been fed and watered in the same manner as the Rambouillets. No ewes had been introduced into these flocks from outside sources for at least four years.

In the light of the diagnosis of vibriosis, the young ewes (2, 3, and 4 years of age) in the Rambouillet band, believed to be most susceptible to the disease, were allotted at random within breeding lines and age groups into five treatment groups; the following treatments were begun on March 31.

1a) No treatment. Fed grain pellets and hay from feed bunks.

2a) 80 mg. chlortetracycline hydrochloride per head per day in mixed-grain, molasses pel-

From the Sheep and Fur Animal Research Branch, Animal Husbandry Research Division, ARS, USDA, U.S. Sheep Experiment Station and Western Sheep Breeding Laboratories, Dubois, Idaho (Hulet, Ercanbrack, Price, Humphrey), in cooperation with the Veterinary Research Laboratory, University of Idaho Agricultural Experiment Stations, Caldwell Branch Station, Caldwell (Frank and Meinershagen). Published with the approval of the director of the Idaho Agricultural Experiment Station, Moscow, as Research paper No. 472.

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* Chlortetracycline as Aurofac-10 and procaine penicillin G and dihydrostreptomycin sulfate as Pro-K-Mycin were supplied through the courtesy of American Cyanamid Company, Pearl River, N.Y.

lets; maintained in a separate pen; fed from bunks.

3a) Two intramuscular injections on consecutive days, of approximately 4 cc. per day of procaine penicillin G in dihydrostreptomycin sulfate solution (200,000 units procaine-penicillin and equivalent of 0.25 Gm. streptomycin base per cc.). Maintained with the controls; fed from bunks.

4a) Same treatment as in 3a, except ewes were kept in an enclosure separate from other lots. Fed from bunks.

5a) Same injections as in 3a plus same amount of chlortetracycline pellets as given in 2a. Maintained with sheep in the main band, all of which received this same treatment; fed on ground.

The young ewes in the Targhee-Columbia band also were randomized within age and line into groups 1b, 2b, and 5b (all other ewes were given the same treatment as 5b); the following treatments were begun on April 2.

1b) Same as in 1a, except fed on ground.

2b) Same as 2a (chlortetracycline), except ewes were maintained with the main band and fed on the ground.

5b) Same as in 5a; penicillin-streptomycin plus chlortetracycline.

Each individual dead immature (premature) lamb or dead full-term lamb (hereafter called immature and dead, respectively) was not examined for the presence of *V. fetus*. Therefore, it was impossible to distinguish between those lambs which were immature or dead due to the organism *Vibrio fetus* and to other causes. The average percentage of 2-, 3-, and 4-year-old ewes dropping dead immature and dead full-term lambs from 1953 through 1957 (both bands) was 3.2 per cent and 3.4 per cent, respectively, as compared with 15.0 per cent and 7.8 per cent, respectively, in the control groups in 1958. The observation of elementary bodies suggestive of the enzootic abortion virus, in smears from the placental cotyledons of 1 lamb, opens the possibility that some of the abortions prior to and during 1958 may have been due to a virus. However, blood complement-fixation tests showed that 98.0 per cent of the control ewes having dead or immature lambs showed titers of less than complete fixation of complement at the 1:8 dilution. This evidence, in conjuction with the isolation and bacteriologic culture of *Vibrio fetus* from nearly every fetus examined, indicates that the abortions encountered in these flocks were preponderantly due to vibriosis. In the control and treatment groups, the distribution of ewes having dead lambs (column 4, table 1) indicates that vibriosis was an important contributing cause of dead lambs as well as immature lambs. For these reasons, all ewes having immature and dead lambs were grouped together in the statistical analysis (column 8, table 1).

Those ewes which had only live lambs, or 1 live and 1 dead lamb, were grouped together (columns 9 and 10, table 1). It is recognized that the ewes which had 1 live and 1 dead lamb may with justification have been classified with those having either dead or immature lambs, as vibriosis may have been the cause of some of these dead lambs. However, none of the control ewes had 1 live and 1 dead lamb and one or more ewes in each group given penicillin-streptomycin injections were in this category. This suggests that 1 of the twins in some of these multiple pregnancies was saved due to the antibiotic, thus giving some reason for classifying this group with those which benefited from the treatment. Further, inasmuch as the numbers were small in this classification, it seemed undesirable to consider them separately in a statistical analysis.

TABLE 1—Comparisons of Antibiotic Treatment Effects on the Number of Ewes Having Dead and Immature Lambs

Group and treatment	No. of ewes	No. of ewes having: Dead, immature lambs	Dead, full-term lambs	Both live & dead lambs	Only live normal lambs	Immature and dead lambs: No.	Immature and dead lambs: %	One or more live lambs: No.	One or more live lambs: %
RAMBOUILLET BAND									
1a, control	84	10	8	0	66	18	21.7	66	78.3
2a, chlortetracycline	82	7	4	1	70	11	13.4	71	86.6
3a, penicillin-streptomycin	86	1	3	3	79	4	4.7	82	95.3*
4a, penicillin-streptomycin	78	3	4	1	70	7	9.0	71	91.0**
5a, penicillin-streptomycin-chlortetracycline	77	5	3	5	64	8	10.4	69	89.6†
TARGHEE-COLUMBIA BAND									
1b, control	96	17	6	0	73	23	24.0	73	76.0
2b, chlortetracycline	86	7	5	0	74	12	14.0	74	86.0†
5b, penicillin-streptomycin-chlortetracycline	90	5	3	3	79	8	8.9	82	91.1*

* $P<0.01$ when compared with control. ** $P<0.02$ when compared with control. † $P<0.05$ when compared with control.

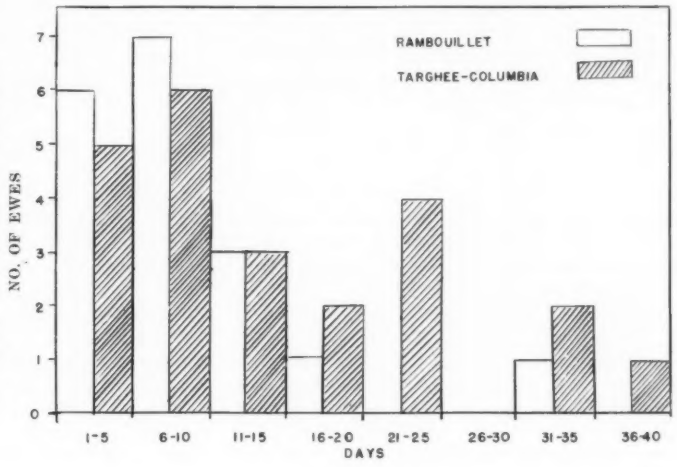

Fig. 1—Frequency distribution of control ewes having dead or immature lambs. This includes all ewes having dead immature lambs and dead full-term lambs, as well as weak lambs which did not survive until ear-tagging or were destroyed intentionally soon after birth. Frequency distribution is shown for five-day intervals; day 1 refers to the first day after the completion of the penicillin-streptomycin treatment of ewes in the other groups.

The statistical significance of each treatment effect was evaluated by the *chi*-square goodness-of-fit test, using the control group as the basis for expected numbers. Differences among treatment groups were tested by a *chi*-square test for independence.

Results and Discussion

Treatments 3a, 4a, and 5a in the Rambouillet band (table 1) each significantly increased the proportions of ewes having at least 1 live lamb ($P < 0.01$, $P < 0.02$, $P < 0.05$, respectively). Chlortetracycline, treatment 2a, appears to have reduced the losses but not to the same extent as the other treatments ($P \cong 0.10$). The ewes in groups 2a and 5a were somewhat slow in taking the full prescribed amount of pellets, and it is probable that a few failed to eat any throughout the entire experiment period.

Among the groups of ewes given antibiotics, there were no significant differences in the proportions of ewes having live lambs. When results of treatments 3a and 4a were pooled and compared with either 2a or 5a, the differences still were not significant. When results of the treatment groups were pooled in any logical combination and compared with those of the control group, the differences were all highly significant. Interestingly, the proportion of ewes having 1 or more live lambs was smaller in group 5a (penicillin-streptomycin + chlortetracycline) than in either of the groups of ewes given only the penicillin-streptomycin injections (3a and 4a), though the differences were not significant. Chlortetracycline when added to penicillin-streptomycin did not increase the effectiveness of the latter under the conditions of this experiment. A logical hypothesis based on previous work [1,2] was that chlortetracycline given daily in addition to initial injections of penicillin-streptomycin would reduce losses even more than the antibiotic alone, presumably because it would prevent subsequent infection. The lack of significant differences between treatments 3a and 5a, and 4a and 5a, might possibly be explained in several ways: *(a)* the ewes given only penicillin-streptomycin injections (3a and 4a) were fed from feed bunks, thus minimizing the possibility of feed contamination with the *V. fetus* organism, whereas those given penicillin-streptomycin plus chlortetracycline (5a) were fed on the ground; *(b)* a large proportion of the susceptible ewes in all groups may have been infected at the time of injection and developed an immunity before the infection was eliminated, thus minimizing the benefit of continual antibiotic feeding; and *(c)* there may have been a slight antagonism among these antibiotics as they affected vibriosis.

Groups 3a and 4a were designed to determine the incidence of reinfection after the effects of the antibiotics had worn off in ewes (exposed to infected and aborting ewes) given penicillin-streptomycin. The lack of significant difference between these two treatments indicates that any losses due to reinfection in group 3a were no greater than those in group 4a (assuming that the

penicillin-streptomycin was equally effective in both groups). This may have been due to improved sanitation (bunk feeding) or immunization, or both, as suggested in the preceding paragraph as two of the possible explanations for lack of differences between 3a and 5a, and 4a and 5a.

Treatments 2b and 5b (table 1) significantly increased the proportion of ewes having live lambs ($P<0.05$, and $P<0.01$, respectively). The difference between the two treatments was not significant.

The frequency distribution (fig. 1), throughout the experiment period, of control ewes having immature and dead lambs indicated that the frequency in either control group reached a peak early in the epizootic, then declined to a minimum near the end of lambing. Obviously, the lower the incidence of vibriosis the lower the savings which may be expected from any treatment.

SUMMARY AND CONCLUSIONS

Vibriosis occurred in both the Rambouillet band and the band composed of Targhee and Columbia ewes at the U.S. Sheep Experiment Station, Dubois, Idaho. Experiments were designed to determine the relative effectiveness of certain antibiotics in reducing losses due to this abortion disease. The young ewes (2, 3, and 4 years of age) were allotted at random (within line of breeding and age group) into a control group and various treatment groups.

Approximately 80 mg. of chlortetracycline per head per day (given in grain pellets) increased the percentage of ewes having at least 1 live lamb to 8 to 10 per cent above that of the untreated controls (differences statistically significant only in the Targhee-Columbia band). Injections of 4 cc. of penicillin-streptomycin on two consecutive days increased the percentage of ewes having at least 1 live lamb 13 to 17 per cent above the untreated controls. When the injected antibiotic was given in combination with 80 mg. of chlortetracycline per head per day in grain pellets, the percentage of ewes having at least 1 live normal lamb was increased 11 to 15 per cent above the untreated controls but was not increased above those given only penicillin-streptomycin. The response of ewes to treatments involving penicillin-streptomycin differed significantly from that of the untreated controls. The differences in response to antibiotic treatments were not significant.

The data show that under the conditions of this experiment the incidence of abortions and dead full-term lambs may be appreciably reduced by penicillin-streptomycin injections at the appropriate stage of infection. They also indicate that chlortetracycline in the feed may, under proper conditions, prove effective as a vibriosis preventive. It is not clear whether the effectiveness of the penicillin-streptomycin injections independent of the chlortetracycline in the feed was conditioned by an immunity developed after infection, the prevention of subsequent infection as a result of bunk feeding, or other undetermined factors.

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Serum Protein Changes in Lambs with Naturally Acquired Nematode Infections

KENNETH L. KUTTLER, D.V.M., and DONALD W. MARBLE, D.V.M.

Reno, Nevada

In view of the nutritional and physiologic changes resulting from internal parasitism, it seems reasonable to expect significant and reproducible changes in serum proteins. Leland *et al.*[9] reported extensive and prolonged hypoproteinemia with hypoalbuminemia and an increase in the *alpha*-2 globulin in calves experimentally infected with *Trichostrongylus axei*. Schumard *et al.*[10] reported an increased albumin-globulin ratio in sheep with mixed parasitic infections. Other workers[12,13] have demonstrated specific, circulating, immune antibodies against some intestinal parasites, suggesting that serum changes may occur, due primarily to the influence of the parasite and not to a secondary effect of other pathologic changes.

Serum protein fractionation by paper electrophoresis is a reliable method for studying changes which occur as a result of various pathologic processes.[2,4] The object of this study was to determine if changes in total protein and the various protein fractions occur in lambs with mixed parasitism. A second objective was to determine if the anticipated serum changes in parasitized animals are associated with the loss of blood or, more specifically, with the presence of intestinal parasites.

Methods

Serum samples were collected from 20 lambs (8 Rambouillets, 1 Columbia, 11 Hampshires) averaging 124 days of age. These lambs showed evidence of clinical parasitism, as determined by visual examination, fecal parasite egg counts, and microhematocrit values. Serum samples were also collected from 11 other lambs (7 Rambouillets, 3 Hampshires, 1 Columbia) averaging 124 days of age and having moderate parasite infections. This classification of "moderate parasitism" was arbitrarily determined on the basis of fecal parasite egg counts and microhematocrit values (table 2). At an average age of 64 days, lambs in both of these groups were placed with their dams on irrigated pastures. Fecal and blood examinations were made periodically and serum samples for test purposes were collected when the desired level of parasitism was reached. The naturally acquired parasitism was predominantly Haemonchus. Identification of Haemonchus eggs on fecal examination was based on size and morphology. Identification of stomach worms as Haemonchus was done at necropsy of lambs dying of parasitism.

Control observations were made on 27 normal, nonparasitized lambs (6 Rambouillets, 9 Hampshires, 2 Columbias, 10 Rambouillet-Hampshire cross) averaging 122 days of age. Eleven were raised relatively free of parasites by weaning at an average age of 91 days while still in drylot. They then were placed on pastures which had not been grazed for a year. The remainder of this group were raised with their dams on drylot and fed alfalfa hay and green pasture clippings from pastures free of livestock.

Anemia was induced in each of 8 lambs (2 Rambouillets, 3 Hampshires, 3 Columbias) by the regular removal of 250 ml. of blood from the jugular vein five days a week for five weeks. The lambs had an average weight of 77 lb. and averaged 189 days of age at the onset of this procedure. These animals, while not parasite free, had fecal parasite egg counts less than 1/20 of those in the moderately parasitized animals and their microhematocrit levels were within the normal range. The progress in producing the anemia and the recovery rate after day 34, when removal of blood was discontinued, is indicated (table 1). Serum samples for total protein determinations and protein fractionations were taken on day 0 when the packed cell volume was 35.6 per cent and on day 34 when it was 19.1 per cent.

Serums were collected from clotted blood samples which had been allowed to stand at room temperature overnight. The serum samples were then frozen at −20 C. for two to six months before protein analysis was done. Recent work[8] indicates that no significant alterations in the serum protein electrophoresis patterns occur in samples frozen for this length of time.

Fecal egg counts were made in a McMaster's egg-counting chamber with some modifications of the technique described by Whitlock.[15] The microhematocrit values used represent the percentage of packed erythrocytes after centrifuging whole blood in capillary tubes for five minutes at approximately 11,000 r.p.m.

Total protein determinations were made on serum samples from the four groups of lambs. The biuret reaction was employed in determining total

From the Agricultural Experiment Station, University of Nevada, Reno, where Dr. Kuttler is associate veterinarian and Dr. Marble is assistant veterinarian.

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TABLE 1—Average Packed Cell Volumes at Various Days in 8 Lambs with Artificially Produced Anemia *

0	3	9	16	23	30	34**	41	51
35.6 ±1.47	24.8 ±1.8	19.5 ±2.3	19.5 ±2.2	19.3 ±2.0	18.5 ±3.1	19.1 ±2.42	26.8 ±1.1	33.9 ±2.1

* 250 ml. of blood was removed from each lamb daily for five days of each week.
** Removal of blood was discontinued after this day.

serum protein.[5-7, 14] Essentially, the method used consisted of adding 2 ml. of a 1:20 dilution of serum to 8 ml. of biuret reagent. The percentage of transmittance was read on a colorimeter set at a wave length of 540 mμ. The percentage of transmittance readings were converted to percentage of protein.[1] A serum standard with a known protein content, as determined by a micro-Kjeldahl test, was used in calibrating the readings obtained by the biuret reaction.

Serum protein fractionation was done on all serum samples from the four groups by means of paper electrophoresis.* After 10 μl. of serum was placed on each filter paper strip, the strips were placed on racks and wetted with a barbital buffer consisting of 1.84 Gm. of barbital and 10.3 Gm. of sodium barbital in 1 liter of distilled water (pH of 8.6). A duostat power source* supplied 15 ma. of direct current for six hours at room temperature. The strips were dyed in bromphenol blue, using the methods described by Block *et al.*[3] Dried strips were analyzed in an intergrating densitometer* using a slit width of 1.5 mm. Percentages of color content of the various protein fractions were calculated from this reading. The protein fractions separated and were recorded in the following order, beginning from the point of origin on the paper strip: *gamma* globulin, *beta* globulin, *alpha*-2 globulin, *alpha*-1 globulin, and albumin.

Total serum protein and serum protein fractions of 20 clinically parasitized, 11 moderately parasitized, and 27 nonparasitized lambs were compared (table 2) by an analysis of variance, single classification,[11] to determine if significant differences occurred. Paired differences of total serum protein and serum protein fractions of 8 animals, before and after anemia was induced (table 3) were analyzed for significance.

RESULTS

The results indicate that significant changes occur in total serum protein and in the serum protein fractions as a result of parasitism (table 2). A decrease occurred in total serum proteins of animals with clinical parasitism. This proved to be significantly different from the moderately parasitized and nonparasitized animals. The difference between moderately parasitized and nonparasitized animals, however, did not reach significance.

TABLE 2—Effects of Parasitism on Sheep Serum Proteins

Condition	No. of animals	Av. age (days)	Packed cell volume	Eggs per gram of feces: Haemonchus	Eggs per gram of feces: Nematodirus	Eggs per gram of feces: Other†	Average electrophoresis values (%): Albumin	*Alpha*-1	*Alpha*-2	*Beta*	*Gamma*	Total protein (%)
Clinically parasitized	20	124	17.0 ±4.3	23,274	339	107	27.4 ±7.1	10.1 ±3.9	23.8 ±3.7	12.1 ±3.2	27.0 ±5.9	3.96 ±0.94
Moderately parasitized	11	124	31.2 ±3.93	4,647	106	257	45.0 ±4.2	6.0 ±1.5	17.0 ±2.1	10.6 ±1.4	21.7 ±3.0	5.35 ±0.42
Nonparasitized controls	27	122	37.1 ±3.7	3	22	0	51.0 ±6.5	4.3 ±1.2	14.1 ±2.6	7.7 ±1.7	22.4 ±4.9	5.81 ±0.54
Value of F							80.05*	26.0*	62.0*	21.6*	6.07*	40.0*
Smallest significant difference							5.1	2.0	2.4	1.9	4.0	0.56

† Other: Trichuris and Strongyloides. * Highly significant.

There was a highly significant decrease in the albumin of blood serum in parasitized animals. A moderate but significant decrease occurred in moderately parasitized animals. A marked decrease occurred in the clinically parasitized animals.

There was a marked increase of all globulin fractions in parasitized animals. The *alpha*-1, *alpha*-2 and *beta* globulins of the clinically parasitized, moderately parasitized, and nonparasitized animals were significantly different. The *gamma*-globulin fraction revealed a significant difference between the clinically parasitized and the moderately parasitized and nonparasitized lambs. The *gamma*-globulin fraction did not reveal a significant difference between

* Spinco model R Paper Electrophoresis Cell, Spinco Duostat, and Spinco model RA Analytrol, manufactured by Beckman Instruments, Inc., Spinco Division, Belmont, Calif.

the moderately parasitized and nonparasitized lambs.

The serum changes occurring as a result of experimentally produced anemia were similar to those seen in parasitized animals, although in most instances they were less severe (table 3). The hematocrit values before and after the production of anemia by regular mechanical extraction of blood generally duplicated the hematocrit values that significant differences existed in the albumin and *alpha*-2 globulin fractions. The albumin was lower and the *alpha*-2 was higher in the parasitized animals than in the relatively nonparasitized, anemic animals. The differences in total serum protein, *alpha*-1 globulin, *beta*-globulin, and *gamma*-globulin fractions did not reach significance. It would, therefore, appear that changes do occur in serum protein

TABLE 3—Effects of Induced Anemia on Serum Proteins

Day	No. of animals	Packed cell volume	Eggs per gram of feces		Average electrophoresis values (%)					Total protein (%)
			Haemonchus	Nematodirus	Albumin	*Alpha*-1	*Alpha*-2	*Beta*	*Gamma*	
0	8	35.6	218	69	52.5	4.4	13.5	7.4	22.6	6.2
		±1.47			±4.1	±1.1	±1.5	±1.5	±3.82	±0.4
34	8	19.1	182	89	37.6	8.6	17.5	11.2	25.1	4.3
		±2.42			±5.8	±2.5	±2.9	±1.8	±1.9	±0.31
Differences					14.9	4.2	4.0	3.8	2.5	1.9
Possibility of error					<0.001	<0.01	<0.01	<0.005	<0.05	<0.001

of the nonparasitized and clinically parasitized lambs. The artificially induced anemia produced a significant decrease in the albumin and significant increases in all globulin fractions. The total serum protein decreased as a result of anemia.

DISCUSSION

The question arises as to whether the differences in total protein and in the various protein fractions in parasitic infections are due to the direct influence of the parasite on the host, or rather represent a secondary effect as a result of the accompanying anemia. Table 4 is presented in an attempt to determine which of the serum changes in parasitized animals might be associated primarily with anemia.

A direct comparison was made between protein values of the relatively nonparasitized animals with induced anemia and the protein values of clinically parasitized animals. An analysis of variance indicates fractions independent of changes associated with anemia.

SUMMARY

Evidence has been presented that total serum protein and all serum protein fractions show significant changes as a result of clinical parasitism in lambs. Significant changes in serum protein also occur with moderate parasitism. In clinically parasitized lambs, the total serum protein is decreased; albumin fraction is decreased; and *alpha*-1 globulin, *alpha*-2 globulin, *beta*-globulin, and *gamma*-globulin fractions are increased.

Similar changes, although less severe, occurred in serum protein of nonparasitized lambs with induced anemia. A significant increase in the *alpha*-2 globulin and a significant decrease in the albumin occurred in clinically parasitized lambs when compared to the nonparasitized lambs with comparable anemia.

TABLE 4—Comparative Effects of Induced Anemia and Parasitic Infection on Serum Proteins

Condition	No. of animals	Packed cell volume	Eggs per gram of feces			Average electrophoresis values (%)					Total protein (%)
			Haemonchus	Nematodirus	Other	Albumin	*Alpha*-1	*Alpha*-2	*Beta*	*Gamma*	
Induced anemia	8	19.1	182	89	0	37.6	8.6	17.5	11.2	25.1	4.30
Parasitic infection	20	17.0	23,274	339	107	27.4	10.1	23.8	12.1	27.0	3.96
Differences		2.1	.....	.....	.....	10.2	1.5	6.3	0.9	1.9	0.34
Value of F		1.54NS	.....	.....	.....	12.9*	0.87NS	17.9*	0.35NS	0.75NS	0.03NS

* Highly significant. NS = Not significant.

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Studies on *Trichostrongylus axei* (Cobbold, 1879). V. Some Quantitative and Pathologic Aspects of Experimental Infections with a Horse Strain in Sheep

S. E. LELAND, Jr., Ph.D.; J. H. DRUDGE, D.V.M., Sc.D.;
Z. N. WYANT, B.S., A.M.; G. W. ELAM

Lexington, Kentucky

A STRAIN of *Trichostrongylus axei*, originally encountered as a natural infection in the horse, was found by Drudge *et al.*[2] to be infective for sheep both directly and indirectly via the calf. The experimental proof of the pathogenic effect of *T. axei* in sheep has been recorded by Gibson[3] with pure infections of ovine origin and by Turner and Kates[17] with nearly pure infections of bovine origin. The quantitative and pathologic features of experimental infections with a horse strain of *T. axei* in rabbits[9] and calves[11] have been reported. The purpose of the present study was to ascertain the lethal larval dose, prepatent period, male/female worm ratio, pathologic effect, and the consequences of reinfection with this horse strain in sheep.

MATERIALS AND METHODS

Nineteen experimentally infected and 4 uninfected sheep (Cheviot ram bred to grade Western ewes), 76 to 657 days old and raised under helminth-free conditions, were used in the present study. In addition, blood determinations made on 31 helminth-free lambs were used to establish normal limits for the cellular components. With the exception of sheep 32 and 71-A, which weighed 131 and 120 pounds, respectively, all others weighed between 50 and 87 pounds when initially infected. For the purpose of convenience, the data have been divided into five groups, with the first four being grouped according to increments of the animals' age at the time of infection. The fifth group was composed of animals 76 to 612 days old when initially infected and for which worm counts were not made. Observations on animals comprising groups I, II, and IV were made in 1954 and those on group III animals were made in 1957. Observations on the animals of group V were made in 1954 and 1957.

The strain of *T. axei* used was of equine origin and was the same strain used in the rabbit[9] and calf[11] studies mentioned previously. The previous animal passages for this strain were: horse-lamb to group I; horse-2 lambs to group II; horse-3 lambs-rabbit-calf to group III; horse-lamb to lamb 311; and horse-lamb-3 rabbits to lamb 32. The previous animal passages of the strain used for the 6 animals in group V varied but were similar to the passages of the strain for the other four groups.

The details of animal facilities, care, diet, larval cultivation, infection techniques, and worm recovery methods, including the artificial digestion technique, were the same as recorded in earlier reports.[9-11] Hematologic procedures were essentially the same as those recorded by Todd *et al.*[16] In addition, the daily food and water consumption was measured in the lambs of group III. The feed and water containers were maintained at the two thirds of capacity level by additions of measured amounts of feed and water. The feed and water containers for each animal were of identical construction.

The method of Reed and Muench[13] was used to estimate the larval $l.d._{50}$.

RESULTS

The $l.d._{50}$ for 19 sheep, 76 to 657 days old when infected, was 580,000 larvae. (Infection is considered as the time at which the infective larvae were administered.)

Clinical symptoms of parasitism were evident at the 250,000 larval dose level, and 1 animal died at this level 57 days after infection.

Although the writers were not able to ascribe an invariable sequence of events with respect to fecal consistency, scouring usually occurred first at about the time the infection became patent in animals which developed clinical cases or died. Scouring was observed to be either continual or intermittent for variable periods of time. If the animal survived, the feces then decreased markedly in quantity and took the form of small, hard, dry pellets. The reduced fecal output was usually accompanied by a marked increase in worm eggs per gram due to the concentrating effect (fig. 4). In some heavily infected fulmi-

From the Department of Animal Pathology, Kentucky Agricultural Experiment Station, Lexington.

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TABLE 1—Worm Counts and Related Data Pertaining to Trichostrongylus axei Infections in Sheep (Groups I–IV)

Animal No.	Age when infected (days)	No. larvae given (thousands)	Days from infection to necropsy	Prepatent period (days)	Worms recovered					In relation to total larvae administered (%)	Male/female ratio
					Abomasal contents	Abomasal digest	Small intestine	Cecum and large intestine	Total		
GROUP I											
41-A	159	50	74	19	22,200	2,816	1,268	0	26,284	52.6	0.68
42-A	161	100	74	26	16,140	983	566	0	17,689	17.7	0.61
41-B	159	250	74	19	22,640	3,498	3,760	0	29,898	12.0	0.40
42-B	161	500	74	26	5,480	750	67	0	6,297	12.6	0.67
GROUP II											
44	243	782	D-81	22	52,150	1,050	100	2,600	55,900	7.1	0.32
439-B	228	1,500	D-20	....	280,400	799	3,550	1,325	286,074	19.1	0.75
GROUP III											
75-A	311	100	91	....	0	0	0	0	0	0	
75	311	250	D 58	....	6,760	120		110	6,990	2.8	0.56
77	323	500	D-38	....	28,800	60		60	28,920	5.8	0.55
77-A	323	750	D-36	....	15,100	130		4,770	20,000	2.7	0.51
78	314	1,000	91	....	9,110	1,160		270	10,540	1.1	0.29
GROUP IV											
311	526	774	D-97	26	35,650	900	5,800	0	42,350	5.5	0.82
32	657	915	72	23	.....	.....	.....	..	.....	....	.....
....	664	116	D-65	....	20,850	9,736	80	0	30,666	3.0	0.73

D = died.

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nating cases the scouring period was not observed, and the animal produced the dehydrated feces in decreasing amounts from patency to death. Other signs of the infection were: (1) reduced feed and water consumption; (2) progressive cachexia; (3) weight loss; (4) listlessness; (5) loss of wool resiliency; and (6) progressive weakness.

Data pertaining to .the various groups are presented (tables 1, 2). The number of worms recovered in relation to the larvae administered was greatest (12.0 to 52.6%) in the younger lambs (group I). With one exception, all other recoveries were less than 8 per cent. The exception was lamb 439-B, from which the greatest number of worms in the present study (286,074) was recovered. This animal died before patency was established and many of the worms recovered were immature forms. Of the 10 animals comprising group II, III, and IV, 7 died. Excluding lamb 439-B, the percentage of worm recovery ranged from 0 to 7.1 per cent. In the 7 that died, the interval between infection and death ranged from 20 to 97 days. The average prepatent period of 12 animals was 23.3 days. The male/female worm ratio was 0.57 for 12 animals.

The number of worms recovered from the contents of the cecum and small and large intestines was usually greater in animals found dead or necropsied *in extremis* than in the other animals of the study.

Sample data showing the influence of infection on the body weight is presented graphically (fig. 1) in terms of the percentage of change from the initial (preinfection) weight. Animals which were given 100,000 or more larvae showed marked reduction in weight, with the body weight being somewhat less than the initial weight for most of the observational period. These postinfection weights were all well below those of the uninfected controls.

Feed consumption was drastically reduced in severely affected animals. In the three fatal cases graphically depicted (fig. 2), feed consumption dropped below measurable amounts before patency and so continued until death. In lamb 78, no measurable amount of feed was consumed between the eighteenth and 32nd day after infection. Thereafter, feed consumption was again evident in this animal but was well below that of the uninfected control for this period.

Water consumption (fig. 2) was also reduced in the severely affected animals,

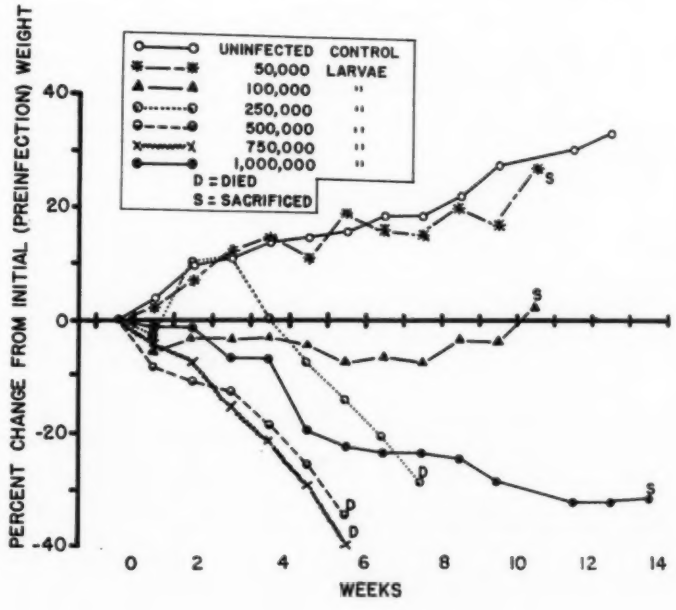

Fig. 1—The influence of Trichostrongylus axei infection on body weight.

though not to the extent that feed consumption was reduced.

Pathology.—The severity of pathologic manifestations were generally, though not consistently, proportional to the number of larvae administered. At the higher larval dose levels, the carcass was in extremely poor condition. Pathologic changes in the abomasal mucosa were first perceptible at the 50,000 larval dose level (74 days postinfection) and consisted of scattered, mildly hyperemic areas. As the number of larvae given was increased above 250,000, the abomasum was more severely affected, and changes ranged from extreme hyperemia to erosion of the mucosa. Occasionally the first few inches of the duodenum were also hyperemic. The degree of abomasitis was progressively decreased in animals which survived beyond the fifth week of infection. Erosion of the mucous membrane was occasionally observed at this time. In 1 animal (yearling 78), circumscribed white areas, 1.5 inches in diameter with raised edges, were observed 91 days after infection. These ringworm-like lesions were similar to those previously reported in horses [12,14] and sheep.[6] However, in infections of similar duration in 3 other animals (44, 311, and 75-A), no ringworm-like lesions were seen.

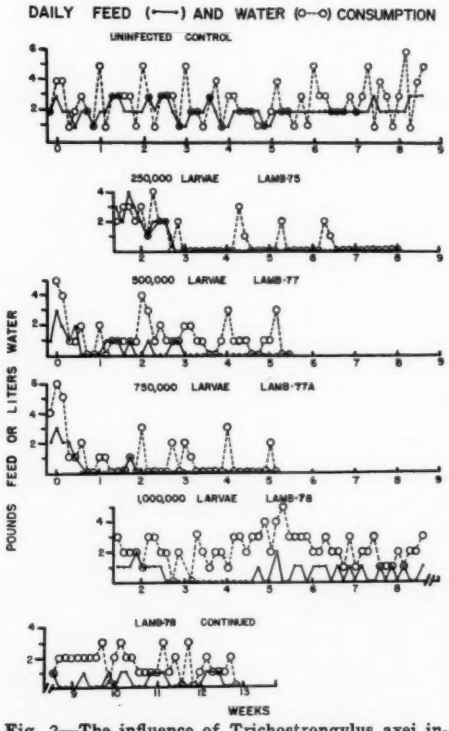

Fig. 2—The influence of Trichostrongylus axei infection on daily feed and water consumption.

TABLE 2—Data Pertaining to Trichostrongylus axei Infections in Sheep (Group V)

Animal No.	Age when infected (days)	Larvae administered		Prepatent period (days)
		Per dose	Total	
47	76	2,500	2,500	25
312	318	5,300	5,300	25
71-A	612	60,000	60,000	20
412	111	35,000		....
	280	87,500	122,500	
	350	42,500*		
514	202	63,000		
	314	1,749,000	1,812,000	25
439-A	228	800,000		24
	640	1,363,000		
	644	1,697,500		
	700	1,749,000	5,609,000	

* 25,400 adult female and 17,100 adult male *T. axei*, same strain, administered with the aid of a dose syringe equipped with a Whitlock nozzle, in 1,700 ml. of diluted abomasal contents from a pure-infection donor calf.

At the higher doses (500,000 or more larvae), the omental fat was usually hard, dull, gritty, and leathery, and gave the general appearance of extensive dehydration. In some animals, the gallbladder was markedly distended. In 4 animals (44, 75, 78, and 311), the livers showed extensive fatty degeneration.

Microscopic examination of sections of the abomasum showed pathologic changes which did not differ essentially from those observed in the rabbit [9] and calf.[11] Considerable sloughing of the surface epithelium was evident. Cells of the lymphoid-macrophage system,[15] lymphocytes predominating, were the principal infiltrating cells; large numbers of these cells were present in the mucosa and occasionally in the submucosa.

Examination of liver sections revealed an extensive fatty degeneration of the hepatic cells in animals 77 and 77-A and in the 4 animals mentioned which had shown fatty degeneration grossly. An example of the extent of liver damage is shown (fig. 5).

No pathologic changes were observed in sections of the lungs, heart, pancreas, or spleen.

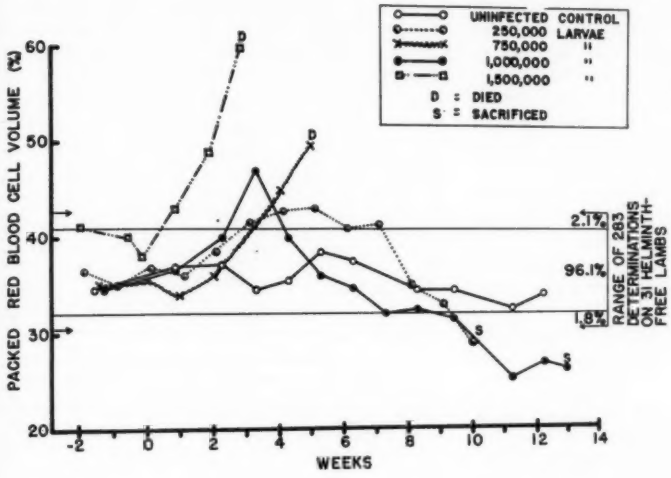

Fig. 3—The influence of Trichostrongylus axei infection on packed red cell volume.

Hematology.—At approximately weekly intervals, packed red blood cell volume (PRCV) determinations were made on 16 of the animals. These determinations covered a period from approximately two weeks prior to infection to 14 weeks or longer after infection. Sample data are presented (fig. 3). In addition, the results of 283 determinations on 31 helminth-free lambs of comparable age are included in figure 3 to establish the normal range.

Six of 7 animals given 750,000 or more larvae and 1 animal given 250,000 larvae showed an elevated PRCV one to six weeks after infection. Five of these 8 animals survived beyond the ninth week postinfection and, in 2 of these 5, the PRCV values dropped into the subnormal range.

Mean corpuscular volume (fig. 4) calculations for the observation period showed no deviation from normal limits. The range for all determinations was 25.3 to 37.7 $c\mu$.

In essence, the hemoglobin and total erythrocyte counts paralleled the changes observed in the PRCV (fig. 4). Total leukocyte counts remained within normal limits except in two instances (fig. 4) where determinations were made on samples from animals *in extremis*, at which time a pronounced increase was observed. Differential leukocyte counts indicated the leukocytosis was largely the result of an increase in neutrophils.

Of 10 animals on which weekly differential leukocyte counts were made, 6 (given 250,000 larvae or more in initial infection) showed an eosinophilia of 10 per cent or higher (fig. 4). Values as high as 16.5 per cent following initial infection were observed. The eosinophilia was usually first evident two weeks after infection and reached its highest level four to eight weeks postinfection, after which the level dropped to within normal limits.

Reinfection.—Three animals were reinfected, and their ages and larval doses are tabulated (table 2). Yearling 32 is not included, since the interval between larval doses was only seven days and it is unlikely that immunity could have developed to any significant degree in this short time. The initial doses of 35,000 and 63,000 larvae in lambs 412 and 514, respectively, were well tolerated, while lamb 439-A (given 800,000 larvae initially) showed severe clinical signs of parasitism. Lamb 439-A slowly but gradually recovered from the initial infection and, at the time of reinfection (412 days later), was in good condition. At the time of reinfection, the 3 animals were either not shedding any worm eggs or showed less than 10 worm eggs per gram (e.p.g.) of feces.

All animals survived and no clinical signs were observed following reinfection, even though 1 animal was given a total of 5,609,000 larvae. Furthermore, weekly worm-egg counts following reinfection were consistently negative in 2 of the animals and were positive in the third (lamb 514) only on rare isolated occasions (10–40 e.p.g.).

An attempt to establish adult worms 70 days after the last larval exposure in lamb 412 (table 2) was unsuccessful. The worm-egg count increased from negative on the day the adults were administered to 280 e.p.g. the following day, but was negative by the fifth day and so remained thereafter for a two-month observation period.

Eosinophilia, which was observed after initial infection, reoccurred more rapidly following reinfection and reached a higher level. It consistently reached its peak 10 days after reinfection, and values as high as 22.5 per cent were recorded. This reoccurrence was also consistent in multiple reinfections in the same animal (lamb 439-A).

DISCUSSION

The l.d.$_{50}$ of 580,000 larvae observed in the present study can be compared with the l.d.$_{50}$ of 500,000 observed for the same strain of the worm in 3- to 5-month-old calves.[11] Although it must be recognized that the age of the sheep when infected covered a wider range (76–657 days), with most infections initiated in animals 200 or more days old, the sheep were rather closely grouped with respect to weight (all but 2 weighed 50 to 87 lb. when initially infected).

Using the same strain of the worm, an l.d.$_{50}$ of 13,000 larvae was observed in 34 rabbits 6 to 12 weeks old.[9]

The equine strain of the present study appears to be more pathogenic in cattle[11] and less pathogenic in sheep than other strains studied,[1,3,8] although the fact that the sheep of the present report were generally older and heavier must be taken into account.

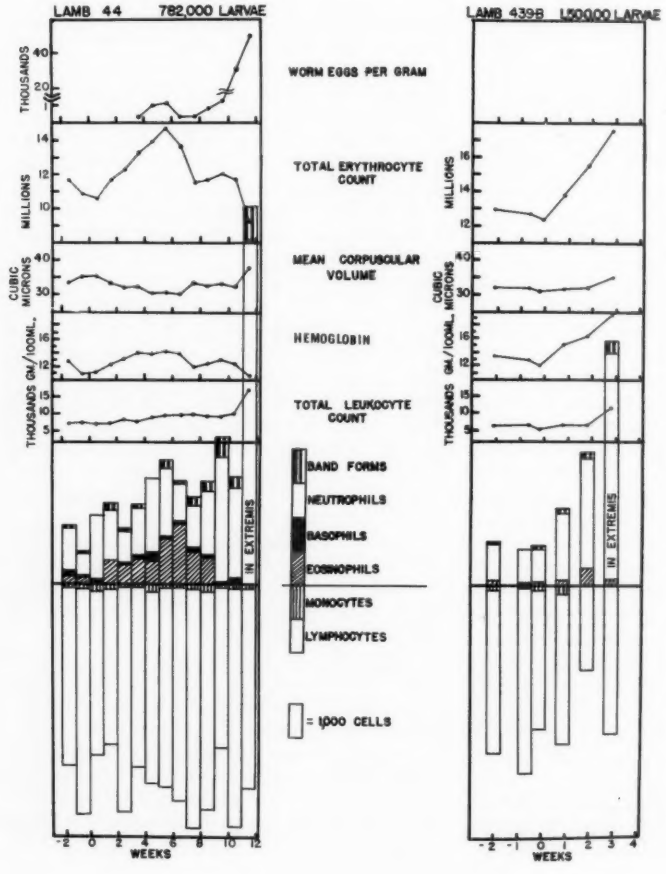

Fig. 4—Hematologic measurements of lambs with Trichostrongylus axei infections.

Turner and Kates [17] reported that of 6 lambs, weighing 25 to 45 lb. and given single doses of 50,000 to 500,000 larvae of bovine origin, 5 died or were killed *in extremis*. Administering 50,000, 75,000, 100,000, 200,000, 300,000, and 500,000 larvae, Kates [8] recorded 91.7, 50.5, 31.6, 10.8, 4.6, and 1.1 per cent worm recovery, respectively, five to six weeks after infection. In the present study, the greatest worm recovery in relation to the larvae administered was in the youngest lambs (group I), with a maximum of 56.2 per cent resulting from a dose of 50,000 larvae.

The greatest number of worms (286,074) was recovered from lamb 439-B, which died before eggs were detected in the feces (20 days after infection). This observation points up two important factors in *T. axei* infections: the worm burden at necropsy is related to the duration of infection, and the pathogenicity of immature stages of the worm is sufficient to cause death. In the calf experiments,[10] serum changes appeared with patency of the worm, indicating that the mechanism responsible is initiated in the immature parasitic stages of the worm.

The interval between infection and death ranged from 20 to 97 days in the sheep and compares with 17 to 39 days in calves [11] and 31 to 72 in rabbits.[9] The average prepatent period in the lambs was 23.3 days and compares with 21.3 days in calves [11] and 22.7 days in rabbits.[9] In sheep, the male/female worm ratio averaged 0.57 and compares with 0.77 in calves [11] and 0.82 in rabbits.[9]

The abstinence from food and water by the lambs in the present experiments was undoubtedly responsible for many of the observed ill effects. However, factors in addition to depression of appetite appeared to be operating in the resulting pathogenic effects of this worm. This was demonstrated by Gibson,[7] who restricted the food intake of worm-free lambs to the amount consumed by lambs infected with *T. axei*. The weight gain of the worm-free group on the restricted diet was intermediate between that of the infected group and a worm-free group on an unrestricted diet. In calves [10] (and in sheep, to be reported elsewhere) infected with *T. axei*, the rather sudden drop in serum protein cannot be explained entirely on the basis of reduced protein intake.

The lesions observed in the present study are, in general, similar to those observed in the calf study.[11] The ringworm-like lesions observed in the horse [12,14] were observed in only 1 yearling (78). Although ovine [5] and bovine [1] strains of *T. axei* in lambs and calves, respectively, have been reported to produce the ringworm-like lesions, with the equine strain this condition has not been observed in calves and was seen only once in sheep. The pathologic manifestations of the equine strain in rabbits [9] on occasion resembles the ringworm-like lesions seen in the horse.

The pattern of changes in the PRCV was essentially the same as in calves,[11] although the occurrence of the pattern was not as consistent in the sheep. Plasma volume

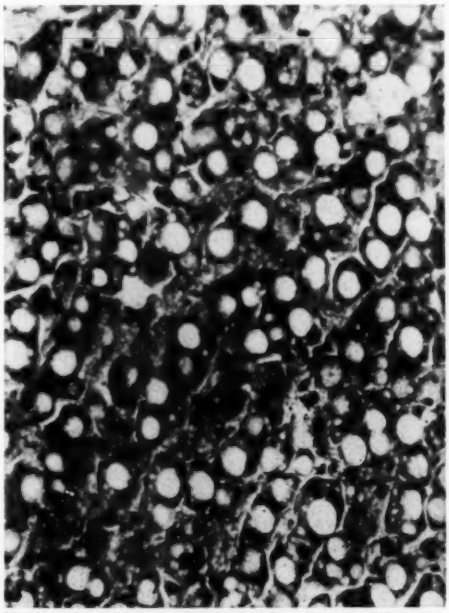

Fig. 5—Liver section showing the extent of damage involved (animal 77).

studies in calves [10] and sheep (to be reported elsewhere) have shown these changes in PRCV to be the result of a reduced plasma volume resulting in a polycythemic hypovolemia which, if the animal survived, developed into an oligocythemic hypovolemia.

Mean corpuscular volume calculations indicate no change in the average cell size occurred as a result of infection with *T. axei*, which is in agreement with Gibson.[5] It is interesting that Todd *et al.*[16] observed

a macrocytic hyperchromic anemia in lambs infected with the medium stomach worms *Ostertagia circumcincta* and *Ostertagia trifurcata* during the interval when the immature forms were establishing themselves in their host.

Whereas the development of an eosinophilia at a predictable point in the infection was one of the most consistent observations in the present study, none of the infected calves[11] in the earlier study showed this characteristic. Rabbits infected with *T. axei* occasionally developed an eosinophilia.[9] Infected calves[11] tended to develop an increase in lymphocytes, but this condition was only observed in 2 lambs of the present study (41-A and 42-B).

The immunity which developed as a result of single infections with 35,000, 63,000, and 800,000 *T. axei* larvae was of a high order as judged by the complete lack of signs and the essentially negative worm-egg counts following the massive challenging doses. That adult worms given to an immune animal failed to become established indicates that the immunity is not only against larval forms. However, in the present study, adults were not administered to a susceptible animal.

The consistently high degree of immunity demonstrated is in contrast to the observations of Gibson,[4] who gave single immunizing doses of 20,000, 15,000, and 10,000 larvae to 1-month-old lambs and observed, respectively, decreased resistance, no effect, and strong resistance to a second infection. It is possible that the mechanism responsible for the production of immunity was not adequately developed or was unreliable in 1-month-old lambs. The development of strong immunity following the recovery of an overwhelming initial infection of *T. axei* (lamb 439-A, in which the prognosis at times was extremely grave following the initial 800,000 larvae) indicates that the resistance mechanism of older sheep is not adversely affected by massive larval doses. It is interesting that lamb 439-A was still immune 412 days after initial infection.

SUMMARY

Nineteen sheep, 76 to 657 days old, were given *Trichostrongylus axei* larvae of equine origin; the l.d.$_{50}$ was 580,000 larvae. In the 7 sheep that died, the interval between infection and death ranged from 20 to 97 days. In 12, the average prepatent period was 23.3 days. The male/female worm ratio was 0.57 for 12 animals.

Clinical signs evident at the 250,000 larval dose level were: (1) reduced feed and water consumption; (2) progressive cachexia; (3) weight loss; (4) listlessness; (5) loss of wool resiliency; and (6) progressive weakness.

Pathologic changes in the abomasal mucosa were first perceptible at the 50,000 larval dose level and consisted of scattered, mildly hyperemic areas. With 250,000 or more larvae, the abomasal lesions ranged from extreme hyperemia to erosion of the mucosa. Ringworm-like lesions similar to those first reported in the horse were observed in 1 yearling. Large numbers of lymphocytes were observed infiltrating the abomasal mucosa and occasionally the submucosa.

An elevated packed red blood cell volume was observed one to six weeks after infection, but it dropped into the subnormal range if the animal survived beyond the ninth week postinfection.

Eosinophilia was observed two weeks after infection. It reached its highest level four to eight weeks after infection, then dropped to within normal limits.

A strong immunity to massive challenging doses of larvae followed recovery from initial infection.

In this study, the equine strain appeared to be less pathogenic for sheep than other strains studied.

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Studies on *Trichostrongylus axei* (Cobbold, 1879). VI. Total Serum Protein, Blood and Plasma Volume, and Electrophoretic Serum Fractionation in Infected and Uninfected Lambs

S. E. LELAND, Jr., Ph.D.; J. H. DRUDGE, D.V.M.; Sc.D.; Z. N. WYANT, B.S., A.M.

Lexington, Kentucky

AN EARLIER REPORT [5] presented results pertaining to blood and plasma volume, total serum protein, and electrophoretic serum fractionation in calves experimentally infected with *Trichostrongylus axei*. The more consistent and prominent serum protein alterations were: (1) an extensive and prolonged hypoproteinemia, measured by grams per 100 ml. of serum and by total grams of circulating protein, and (2) a hypoalbuminemia with an increase or maintenance of the *alpha*-2-globulin level and, in general, a decrease of the other globulins, all of which were evident in percentage composition, concentration (Gm./100 ml. of serum), and grams of total circulating component. The sequence of events with respect to blood and plasma volumes in infected calves was the development of polycythemic hypovolemia* which, if the animal survived, developed into oligocythemic hypovolemia.

This report concerns an identical study in which only the host was changed; *i.e.*, lambs were used in place of calves. The purpose of the study was (1) to investigate the changes in serum protein components which occur as a result of experimental infection of lambs with *T. axei*; (2) to study these changes progressively from a preinfection period through the course of the infection; (3) to evaluate the influence of this infection on the total circulating serum, plasma, and cellular volumes of the blood; and (4) to compare the changes that occur in the bovine and the ovine host, using the same strain of worm in the absence of other worm species. The experimental design consisted of pre- and postinfection measurements on the same lamb, as well as parallel measurements on helminth-free control lambs.

MATERIALS AND METHODS

Animals and Animal Facilities.—Twelve helminth-free, crossbred lambs (grade Western ewes-Cheviot ram) were used in this study. They were inoculated with *T. axei* larvae and 2 served as helminth-free controls. At the time of larval inoculation, the lambs were 3½ to 5½ months old. They were individually housed in pens with expanded metal floors and fed a previously described [5] pelleted ration.

Infection Technique.—The larval culture, the counting, the administration methods, and the strain of *T. axei* (equine origin) were identical with those used in the calf experiments.[5] Larval doses were as follows: Lambs 75A and 42A were given 100,000 larvae; lamb 75 was given 250,000; lambs 77 and 42B were given 500,000; lambs 77A, 44, and 311 were given approximately 750,000; lamb 78 was given 1,000,000; and lamb 412 was given a total of 122,500 larvae in two doses. Lambs 71 and 4M were uninfected, helminth-free controls. Worm counts, pathologic changes, duration of infection, feed and water consumption, and other related information for these animals have been recorded elsewhere.[7]

Serum and Blood Analyses.—Total serum protein (biuret and refractive index methods), electrophoretic fractionation and mobility (Perkin-Elmer Model 38), and blood and plasma volume (dye method) were determined in a manner already described.[5]

RESULTS

Hypoproteinemia was observed in the sheep of the present experiments although not as consistently from animal to animal as in the calf experiments.[5] Three of 10 sheep (44, 77, and 78) on which total protein determinations were made developed hypoproteinemia of less than 5 Gm. per 100

From the Department of Animal Pathology, Kentucky Agricultural Experiment Station, Lexington.

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* The descriptive terminology concerning blood and plasma volume used in this report is as defined in Best and Taylor.[2]

ml. of serum (fig. 2). The hypoproteinemia was observed only with the administration of 500,000 or more larvae.

Examples of the data obtained from electrophoretic serum fractionation are shown (fig. 1, 2). A series of electrophoretic patterns shows the deviations of an infected animal's pattern (75) from those of the preinfection period and a control lamb (71) for the corresponding period (fig. 1). At 23 days postinoculation, increased *gamma*-1-globulin was recorded. At 30 days, the *gamma*-1-globulin was still increasing and was accompanied by a marked *gamma*-2-globulin increase and a marked reduction in the albumin component. At 37 days, the *gamma*-1-globulin was decreasing, and the *gamma*-2-globulin was still increasing. The albumin still was reduced. At 44 days, the *gamma*-1-globulin still was decreasing, and the *gamma*-2-globulin still increasing. The albumin was increasing. At 51 days, the *gamma*-1-globulin was nearly absent, with the *gamma*-2-globulin unchanged from the 44th day. The albumin still was increasing but was not at the preinfection or control level. At 58 days, the animal died as a result of the infection.

Four animals (75, 77, 78, and 311) which had received 250,000 or more larvae had essentially the same sequence of serum protein changes described previously.

Variable serum protein changes occurred in the remaining animals. One animal given 750,000 larvae (77A) did not show the *gamma*-2-globulin increase but showed the *gamma*-1-globulin increase and marked albumin reduction. Animal 412 (122,500 larvae in two doses) showed only the *gamma*-2-globulin increase. One animal (42B) given 500,000 larvae also showed only a slight increase in the *gamma*-1-globulin in the only postinfection measurement made (24 days after infection). Two animals (42A and 75A) which were given only 100,000 larvae showed essentially no electrophoretic pattern changes from those for the preinfection or corresponding periods in the controls.

Sample quantitative results of electrophoretic serum fractionation are presented (fig. 2) in terms of percentage composition, concentration (Gm./100 ml. of serum), and absolute values (total grams of serum protein). All three methods of expression show that changes in the *gamma* globulins occurred following infection. A comparison of the absolute values of the uninfected control (71) with those of lamb 78 revealed a gradual decrease in total grams of serum protein in the latter, whereas, as would be expected in a growing animal, a gradual increase was observed in the uninfected control. This decrease in the infected animals was largely the result of a decrease in the albumin fraction.

Statistical analysis (standard error of the difference between the means) of the electrophoretic mobilities of the individual serum components showed no difference from preinfection to postinfection or between infected and uninfected animals. Thus, a condition of dysproteinemia resulted following infection since no evidence of qualitative serum protein change was observed.

Blood and plasma volume determinations were made on 6 animals (71, 75, 75A, 77,

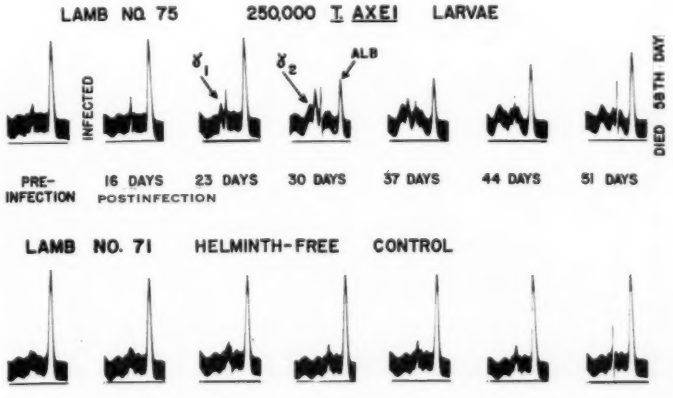

Fig. 1—Sample electrophoretic patterns (descending limb) of serum from an exposed lamb and an unexposed control lamb for the corresponding periods. Analyses were made at a protein concentration of 2 per cent, in veronal-sodium veronal buffer at pH 8.6 with a potential gradient of 7.9 volts per centimeter for two hours.

77A, and 78). The influence of infection on the various volumes in a lamb with a fatal case (77A) are shown (fig. 3). Lines were fitted to control values (uninfected control lamb 71), and these lines superimposed (fig. 3, 4). Lamb 77A (fig. 3) showed a progressive reduction in the blood volume following infection. This decrease was found to be due to a reduction in the plasma portion with the cellular volume remaining normal. The net effect of this situation is hemoconcentration or, more precisely, polycythemic hypovolemia.

The progress of a lamb (78) which survived beyond the thirteenth week was charted (fig. 4). Through the fourth week, the pattern was much the same as for the lamb with a fatal case, although values were lower. Thereafter, the cellular volume began decreasing, indicating that the hemodilution was the result of an absolute decrease in the cellular volume.

Lambs 75, 77, 77A, and 78 (250,000 or more larvae) showed, depending on the duration of infection, the previously described sequence of events with respect to blood, plasma, and cellular volumes.

Thus, the sequence of events with respect to the blood volumes following experimental infection with *T. axei* is the development of a polycythemic hypovolemia which, if the animal survives, develops into an oligocythemic hypovolemia. In the transition from the former to the latter, a condition of simple hypovolemia manifested itself.

DISCUSSION

Mechanisms which lead to a reduction of serum protein [4] are (1) diminished formation as the result of (a) deficient protein intake, (b) disease of the liver, probable formation site of albumin and *alpha* and *beta* globulins,[9] and (2) excessive loss due to nephrotic disease. Lesser reductions are found in many chronic infections, instances of impaired absorption of protein, chronic diarrhea, and open suppurative draining wounds.[4]

Feed consumption and corresponding protein intake were drastically reduced in lambs [7] and calves [6] severely affected by *T. axei* infection. Factors in addition to deficient protein intake, however, appear to have contributed to the hypoproteinemia.[5] In heavily infected lambs, extensive fatty degeneration of the hepatic epithelial cells was observed.[7] This probably impaired protein synthesis. Another means by which plasma protein was reduced may have been by loss through the alimentary tract via the abomasal lesions. Protein loss via the kidneys was not determined in the lambs, but in the calf experiments [6] urinalyses were negative for albumin.

The results of the electrophoretic serum fractionation in the present sheep study provide an interesting variation from the

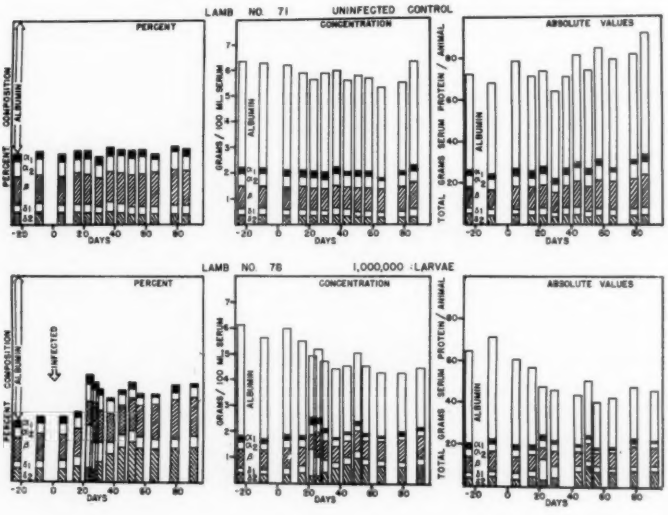

Fig. 2—Sample data from electrophoretic serum fractionation.

calf study. Of more than 110 electrophoretic analyses of serum from infected calves,[5] only two analyses—a day apart and in the same animal—showed an increase in the *gamma* globulins comparable to that seen in the lambs of the present study. However, only one of 70 analyses in this lamb study showed an increased *alpha* globulin comparable to that seen in the calf [5] study. Thus, the two hosts display, with respect to serum proteins, a distinctly different reaction to experimental infection with the same strain of *T. axei*.

The situation as seen in the lambs, *i.e.*, the drastic reduction in albumin and the minor changes of *alpha* and *beta* globulin, could tentatively be explained as a result of primary or secondary liver involvement since this organ is the probable formation site of serum albumin and *alpha* and *beta* globulins.[9-11] The accompanying increases in the *gamma* globulins could be the result of (1) an alteration in the rate of synthesis, (2) an antibody formation against (a) the worm, (b) invading microorganisms which have gained entrance via the abomasal lesions, or (c) the metabolic products of the worms or microorganisms, and (3) a compensatory reaction to restore serum osmotic pressure which would be greatly reduced as a result of the low albumin concentration.

Although no statement of finality can be made at this stage with regard to the mechanism involved in the observed serum protein changes, a few points of interest have evolved. Liver involvement cannot be accepted unequivocally as the sole cause for the described reductions in albumin and *alpha* globulin in lambs, since in calves where only minor liver damage was seen [5,6] the *alpha* globulin was greatly increased but the albumin was markedly reduced. One might postulate, in the case of the calves, that any damage to the liver was sufficient to reduce the production of albumin but stimulated the over-production of *alpha* globulin and that when the liver damage becomes extensive the picture as seen in lambs would predominate. The rare instances mentioned previously, where the lamb and calf situations were reversed, would be compatible with this hypothesis since the liver involvement might have been just sufficient to produce the respective changes. The possible influences of malnutrition on the liver, of a toxin liberated by the worm,[3] or of toxic substances released by the extensive tissue destruction also may be contributing factors.

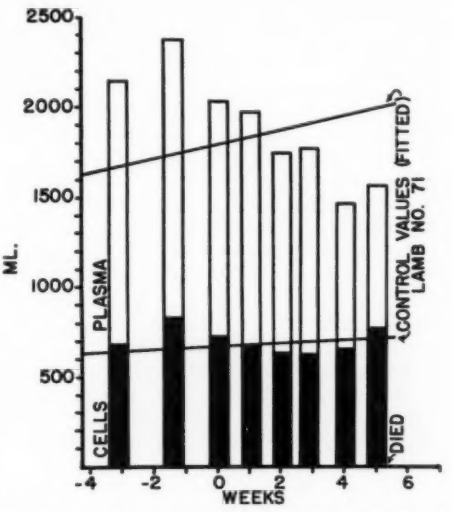

Fig. 3—The influence of infection on the blood and plasma volumes in a lamb (77A) which died after being given 750,000 larvae.

Since the synthesis of *gamma* globulins is not believed to occur to any extent in the liver,[10,11] increases in these serum components could occur in the presence of liver impairment either as a compensatory or altered rate of synthesis mechanism. However, marked increases in the *gamma* globulins occurred only in the lambs following infection and were essentially absent in the calves.[5]

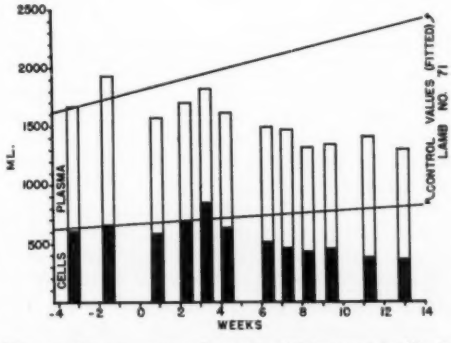

Fig. 4—The sequence of events with respect to blood and plasma volumes in a lamb which survived 13 weeks after exposure.

The formation of antibodies with protective qualities did not appear to be a factor associated with the observed serum protein increases since lambs with extremely high *gamma*-globulin levels succumbed to the infection. Furthermore, lambs which had low-level infections (100,000 larvae) or which had spontaneous loss of worms showed no increase in *gamma* globulins. This would tend to support the hypothesis that a reaction to injury or shock-type [8] of response existed in the lambs which manifested the observed serum protein increases. Further, clinical signs of parasitism were evident at the 250,000 larval dose level and the serum protein changes were generally associated with the degree of clinical manifestations.

Following infection, the sequence of events with respect to blood, plasma, and cellular volumes was essentially the same as that observed in the calf study;[5] *i.e.*, the blood volume began decreasing as a result of the reduction in plasma volume while the cellular volume remained normal. The net result was an increase in the cell to plasma ratio (relative polycythemia). Four weeks after infection, the blood volume was still considerably less than the control and, in addition, the cell volume began to decrease, resulting in absolute oligocythemia during the latter part of the observation period.

The possible mechanisms which could bring about the observed changes in the various blood volumes have been described in detail [5] and will be repeated only briefly here. Factors contributing to the reduced plasma volume were (1) negative water balance brought about by reduced intake and increased loss associated with the diarrhea and the loss via the abomasal lesions, (2) reduced plasma protein brought about by a reduced rate of synthesis and loss via the abomasal lesions, and (3) reduced protein intake. In the present lamb study, as in the calf study,[5] the reduction in cellular volume observed toward the end of the observation period may be the result of (1) prolonged anhydremia, (2) the anemia associated with long standing infections where increased blood destruction may not be the causative factor but, instead, a disturbance in iron metabolism accompanied by a reduced plasma iron and iron-binding protein, (3) a reduction in the erythrocyte life span of sufficient magnitude that the bone marrow cannot compensate,[1] (4) a specific nutritional deficiency, (5) autoimmune anti-red blood cell antibody formation,[12] and (6) toxins resulting from *T. axei* infection which interfere with the blood-forming cells.[3]

SUMMARY

Twelve crossbred lambs (grade Western ewes—Cheviot ram), raised helminth-free, were used in the study. Ten of these lambs were inoculated with *Trichostrongylus axei* larvae and 2 served as helminth-free controls. At the time of larval inoculation, the lambs were 3½ to 5½ months old. The dose range was 100,000 to 1,000,000 *T. axei* larvae. In all but 1 lamb, a single larval dose was given. Marked deviations from the preinfection or control values for serum proteins and blood and plasma volumes were obtained in lambs infected with 250,000 or more larvae.

Serum protein alterations were (1) hypoproteinemia as measured in grams per 100 ml. of serum and total grams of circulating protein and (2) hypoalbuminemia with an increase in the *gamma* globulins, all of which were evident in percentage composition, grams per 100 ml. of serum, and grams of total circulating component.

The sequence of events, with respect to blood and plasma volume alterations in infected animals, was the development of polycythemic hypovolemia which, if the animal survived, developed into an oligocythemic hypovolemia. At a point in the transition from the former to the latter condition, a state of simple hypovolemia existed.

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Some Factors that May Influence Hog Cholera Transmission

RICHARD W. HUGHES, D.V.M., M.S., and DONALD P. GUSTAFSON, D.V.M., Ph.D.

West Lafayette, Indiana

RECENT WORK by Shope [30,31] showing the swine lungworm to be a reservoir of the hog cholera virus (HCV) has renewed interest in the possibility of controlling hog cholera by limiting its reservoirs and vectors. The recognition of such a reservoir, while essential to the control of hog cholera, still seems to leave the mode of spread of the disease between farms in question. These studies were designed to explore several facets of the hog cholera transmission problem to find some clue that might lead to an understanding of how dissemination takes place. The problem was approached by investigating some of the natural routes of exposure of swine to HCV and by studying some of the possible carriers of the virus. In conjunction with these studies, observations were made on the influence of *Salmonella choleraesuis* on susceptibility of swine to the virus.

LITERATURE REVIEW

During an epizootic of cholera, the disease appears to spread quite rapidly, in an erratic fashion, to a majority of the nonimmune herds in the area. A search for some obvious contact between the originally infected herd and the newly infected ones is often unsuccessful and the mode of transmission becomes a matter of speculation. The results of transmission experiments have not always supported these speculations.

With regard to air-current or aerosol transmission, the Indiana Experiment Station reported in 1921 [2] and again in 1922 [3] that, in a closed house, a complete cheesecloth screen protected swine on one side of the screen against cholera-infected swine on the other side. An incomplete cheesecloth partition did not protect controls. Also, this record reported positive transmission in a pasture in which spaces of 4 and 8 ft., respectively, separated sick animals from the controls.

Curphey [5] described an experiment in which 15 susceptible pigs were kept for eight weeks in a fly-proof quarantine pen located in a barn which contained pigs affected with hog cholera. None of the 15 pigs contracted hog cholera during that period.

The aerosol dispersal experiments of Schwarte and Mathews [28] involved the use of lyophilized hog cholera virus. Two pigs were exposed for ten minutes by being put in an isolation room in which lyophilized virus had been exploded 15 and 30 minutes previously. The pigs were covered so that only their snouts were exposed directly to the room. Both pigs developed cholera after the exposure. The authors concluded that this might be a method by which cholera could be spread in nature.

Because blood, secretions, and excretions of cholera-affected swine are known to be infectious, both sucking and nonsucking insects which feed on these substances have been considered to have a possible role in the dissemination of hog cholera. The results of experimental work using insects as vectors are conflicting. Bernazky,[7] in 1910, and Todoroff,[34] in 1936, concluded from their experiments that the hog louse *(Hematopinus suis)* was capable of transmitting cholera from sick to healthy pigs. On the other hand, Dorset *et al.*,[12] in 1918, discontinued their work with the hog louse because all transmission attempts were unsuccessful. In this same report, Dorset presented evidence that the housefly *(Musca domestica)* and the stablefly *(Stomoxys calcitrans)* were both capable of taking up the virus from infected secretions and blood and harboring it for several days. Only the stablefly, however, seemed capable of actually transmitting the disease from sick to healthy pigs. The Indiana Experiment Station, in 1919,[1] reported no success in transmitting hog cholera by means of stableflies.

Wild and domestic mammals have been suggested as possible carriers of hog cholera; however, attempts to transmit the disease to swine from exposed mammals have generally been unsuccessful. Jacotot [19–21] studied the persistence of the cholera virus in many species of mammals. He showed that, with a few exceptions, HCV persisted in most of the wild and domestic mammals for a few days after it was injected into them. He presented little evidence to establish whether these same animals could transmit the disease from sick to healthy pigs. TenBroeck [33] and Dorset *et al.*[11] found that wild rats were not able to harbor the virus or transmit it to susceptible pigs. Popovic [25] showed that after exposing dogs, by injection or *per os*, the blood and excrement of these dogs did not infect swine. Sheep and goats have been shown by Jacotot,[19–21] Jezic,[22]

From the Department of Veterinary Science, Agricultural Experiment Station, Purdue University, West Lafayette, Ind.

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Zichis,[35] and by Hupbauer and Skokovic[13] to be able to harbor the virus for as long as three weeks. Both Zichis and Jacotot reported transmission by cohabitation of sheep with hog cholera-infected swine, but Hupbauer and Skokovic reported negative results with cohabitation trials. Because of the relatively confined conditions under which they are maintained, sheep probably do not play an active role as vectors of cholera. In recent years, domestic rabbits have been shown capable of harboring and multiplying HCV through numerous artificial passages[4, 6, 34, 24]; however, TenBroeck[33] could not demonstrate the presence of the virus after intravenous or intraperitoneal inoculation in rabbits. Hagan[17] does not believe such multiplication occurs in rabbits under natural conditions.

Among the more likely vectors of hog cholera are wild birds. Dorset *et al.*[11] reported that susceptible pigs were exposed for long periods to pigeons which passed daily from a pen containing swine dying of hog cholera to the pen containing susceptible pigs only 10 ft. away. None of the susceptible pigs developed cholera after several months of such indirect contact. The buzzard was studied by Dawson *et al.*[10] in 1914. They found that after buzzards had been fed on the flesh of hogs affected with cholera, the droppings of these birds did not produce cholera when fed to susceptible swine. Jacotot[20, 21] found that after the inoculation of HCV into chickens and pheasants, the blood of these birds was not infectious for swine. When pigeons were similarly treated, the blood was infectious for swine in only one of several trials.

One of the largest gaps in the published data on hog cholera transmission concerns the routes, other than parenteral, by which the virus is infective. Schwarte and Mathews[27] produced hog cholera by injecting the virus into the nasal cavities and into the trachea of swine. Slavin,[32] however, had no success in causing the disease when the virus was introduced into the nasal cavity or the conjunctival sac. According to the experiments of Slavin,[32] Geiger,[15] and David,[9] the oral route appears to be an uncertain infectious channel. All three workers reported great difficulty in infecting pigs when the virus was given orally. David reported that pigs often did not become infected when they were allowed to feed from troughs smeared with virulent virus in blood. Dorset *et al.*[11] related that when fed and scattered in pens, the freshly collected secretions and excretions of cholera-sick pigs were, as a rule, noninfectious.

One of the most puzzling observations concerning hog cholera transmission is the erratic course the disease follows during an outbreak. Hogs that should be highly susceptible to cholera on one farm may not contract the disease even though practically all surrounding farms are affected. This observation, along with the irregular results of transmission experiments, suggests the possibility that an unknown factor may be necessary before HCV is infective by a particular route of exposure in the dilution presented by a passive vector. No reports of such an adjuvant factor have been found in the literature.

MATERIALS AND METHODS

Salmonella choleraesuis, S-105.—The strain of *S. choleraesuis,* S-105, was isolated in pure culture from the blood of a hog having clinical signs and lesions suggestive of hog cholera. According to animal inoculation trials, the blood did not contain HCV. Pigs fed 1.0 ml. of a 24-hour culture of this organism developed transient fever and anorexia, which lasted three to four days. Dilution trials showed that 1.0 ml. of a 10^{-8} dilution of a 24-hour culture of S-105 could be fed to young pigs without causing any noticeable signs except transient fever in an occasional pig.

The characteristics of *Salmonella choleraesuis* S-105 are:

Morphology:	Slender gram-negative rods measuring approximately 0.6 x 5.0 μ.	
Reactions:	Dextrose	acid and gas
	Lactose	negative
	Sucrose	negative
	Maltose	acid and gas
	Mannitol	acid and gas
	H_2S	negative
	Indol	negative
Motility:	Positive	
Antigens:	Group C; somatic 6 and 7; flagellar 1 and 5	

Hog Cholera Virus.—The Carl Johnson and the Floyd Chapman strains of HCV referred to in the procedures were isolated from their respectively named farms during a rapidly spreading outbreak of hog cholera in Carroll County, Indiana. The identification of these agents as hog cholera was made by swine-inoculation trials. The virus samples used in the experiments were bacteriologically sterile, defibrinated blood which had an infective titer of at least 10^6 infective doses per milliliter.

Swine.—All swine used in these experiments were obtained from the Purdue University Veterinary Science Department's disease evaluated swine herd. All swine on this farm are Yorkshire-Chester White-Tamworth crossbred, and have been shown to be susceptible to hog cholera and *S. choleraesuis* infections.

Isolation Cans.—The isolation cans referred to in these experiments are larger, rectangular modifications of the isolation cans described by Haelterman.[16]

Houses for Sparrow Transmission Experiment.—These screened, movable, wooden houses had dimensions of 6 by 6 by 8 ft. They were connected to each other by a 2 by 2 by 6 ft. screened flyway located 4 ft. from the ground. To prevent rodents from burrowing under the houses, 18-inch wide hardware cloth was nailed to the bottom of the houses and buried under the ground. A gate 3 ft. high, placed inside each house 3 ft. from the door, allowed the pigs to be fed and watered without persons having direct contact with them.

English Sparrows.—English sparrows *(Passer domesticus)* were trapped on the Purdue University veterinary science farm. Approximately equal numbers of mature males and females were used.

Manure Worms.—Manure worms *(Heliodrilus sp.)* were obtained from manure piles on the grounds of the Purdue University Veterinary Science Department.

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EXPERIMENTAL PROCEDURES

PART I—STUDIES OF SOME NATURAL ROUTES OF EXPOSURE OF SWINE TO HOG CHOLERA VIRUS

Experiments were designed to investigate the importance of some natural routes of exposure in the transmission of HCV. Routes studied were the oral, conjunctival sac, abraded skin, and unbroken skin. The oral pathway was examined in more detail than the others because it seemed to be the most probable route of exposure; *i.e.*, through the ingestion of infected material brought by vectors.

Parallel experiments using *S. choleraesuis* plus HCV were designed to determine if the presence of these bacteria affected the establishment of hog cholera.

The Carl Johnson strain of HCV, isolated from a field outbreak of hog cholera, was used throughout the group of experiments in part I.

Experiment 1.—the Oral Route.—In two trials, spaced one month apart, a total of 20 pigs were exposed to HCV by mixing virus-containing material in 8 oz. of each pig's daily ration of milk. In the first trial, 6 pigs were exposed. Two pigs each were given 1.0 ml. of undiluted HCV, 10^{-1} and 10^{-2} dilutions. The second trial was similar to the first, except that the series of log dilutions was extended to 10^{-6}, and 14 pigs were used. One of the 2 pigs at each concentration level received, in addition to the HCV, 1.0 ml. of a 10^{-3} dilution of 24-hour broth culture of *S. choleraesuis* mixed with the virus-milk mixture.

Eleven other pigs served as experimental controls for the two trials. Five of these were inoculated intramuscularly with 1.0 ml. of dilutions, from 10^{-4} through 10^{-6}, of the HCV used in the oral exposure. Four pigs were exposed orally to 1.0 ml. of a 10^{-2} dilution of the *S. choleraesuis* culture described above. In each trial, 1 pig remained untreated and served as an environmental control.

Each pig was maintained in a separate isolation can. Observations and body temperatures were recorded daily. White blood cell counts were made prior to exposure to the virus and again ten days later. Possible accidental transmission of the disease was controlled by rigid disinfection procedures, the presence of untreated control pigs, and by minimizing contact with the pigs. Observations were continued for three weeks. At three weeks, all pigs in good health were challenged intramuscularly with 1.0 ml. of a 10^{-3} dilution of the virus strain used in the oral exposure.

Experiment 2—the Conjunctival Sac.—Four pigs were exposed to HCV by placing virus-containing material in the conjunctival sac. One of these pigs received 1 drop (1/20 ml.) of undiluted HCV in the conjunctival sac of one eye, while another received 1 drop of undiluted HCV plus 1 drop of a 24-hour culture of *S. choleraesuis* in one eye. The other 2 pigs were exposed similarly except that a 10^{-1} dilution of HCV was used.

Experiment 3—Topical Exposure of Abraded Skin.—Two pigs were exposed to hog cholera by way of abraded skin. An area of skin on the back of each pig's neck was prepared by dry shaving. This resulted in obvious abrasions of the skin but no gross bleeding. One pig received 0.1 ml. of undiluted HCV placed carefully on this spot, and the other pig received the same amount of virus plus 0.1 ml. of a 10^{-3} dilution of a *S. choleraesuis* culture.

Experiment 4—Topical Exposure of Unbroken Skin.—Six pigs were exposed to hog cholera as in experiment 3, except that the virus material was applied to apparently unbroken skin instead of abraded skin. Three of these pigs were exposed to undiluted HCV only, while the other 3 were given a mixture of undiluted HCV and *S. choleraesuis* as in the previous experiment.

PART II—STUDIES OF SOME POSSIBLE VECTORS OF HOG CHOLERA

The possibility has been discussed that HCV can be spread by the wind as an aerosol suspension; however, published evidence that such occurs in nature was not found. One of the following experiments was designed to explore the possibility of natural aerosol transmission of hog cholera.

The free-flying birds seem to be among the more probable of the animal vectors of hog cholera because of the manner in which they travel from farm to farm. From the many species of birds available for study, the English sparrow *(Passer domesticus)* was selected because of the enormous population it represents among the wild birds, because it lives in close contact with swine, and because the spread of this bird to all parts of the United States during the last half of the nineteenth century, as described by Barrows,[5] coincides in many ways with the rapid advances made by hog cholera during that same period, as described by Salmon.[26] No reports of English sparrows being used in transmission studies were found in the available literature.

Since hog cholera outbreaks are more common during the summer months when flies are most abundant, an experiment considering stableflies and houseflies as vectors of this disease was included. Earlier investigators disagree as to the importance of flies in the transmission of hog cholera.[1,12]

The common belief that earthworms can harbor and transmit hog cholera stimulated the study of one genus of these worms, the manure worm, to find if it could harbor HCV.

Salmonella choleraesuis was incorporated into most of these experiments to determine if its presence had any influence on the success of hog cholera transmission by vectors.

Experiment 5—Aerosol Vection.—Eighteen isolation cans were connected in pairs (fig. 1). A susceptible pig was placed in each can B, and a pig which was inoculated with 1.0 ml. of undiluted Carl Johnson HCV was placed in each can A. As the cans were arranged for this experiment, filtered air was forced by positive pressure through can A, which contained the infected pig, and out the exit tube which was connected to the inlet tube of the adjoining can B, which contained the susceptible pig. This served as the only source of air

for can B; therefore, if any HCV became air-borne, it would be forced out of the contaminated can and into the can containing the sueceptible pig.

After one week, a susceptible pig was placed in each of the A cans, and the experiment was allowed to continue for two more weeks. This pig served as a control to determine if the inoculated pig in can A could transmit cholera to a susceptible pig by direct contact. The experiment was allowed to run a total of three weeks, and any pig that did not develop cholera during this exposure period was challenged by placing it in the adjoining can containing the infected pigs.

In five of these nine pairs of cans, in addition to the described procedure, *S. choleraesuis* was incorporated as follows. In three of the five pairs of cans, 1.0 ml. of a 10^{-3} dilution of a 24-hour broth culture of *S. choleraesuis* was given orally to the pigs in cans A, four days before the injection of HCV was given. In the remaining two pairs of cans, the susceptible pigs of cans B were given 1.0 ml. of the *S. choleraesuis* suspension orally on the same day the pigs in cans A were inoculated with HCV.

Experiment 6—the English Sparrow.—This experiment was designed in a rather simple manner in an attempt to simulate field conditions as much as possible; however, measures were taken to eliminate or control variables other than the sparrow. The human factor was controlled by employing rigid disinfection procedures, regulating the order of entrance into the houses, and by minimizing contact with the pigs. The two trials were conducted during winter and early spring months in order to minimize insects as complicating factors.

Trial 1.—Portable isolation houses were arranged in the manner as shown in figure 2. House A, which originally contained 4 pigs, served as a source of infection. One of these pigs was injected with 1.0 ml. of Carl Johnson HCV and the other pigs became infected by contact. Additional susceptible pigs were added to house A from time to time to keep the disease present in all stages of development. House A was connected by a 6-ft. flyway to house B which contained 3 susceptible pigs and 1 serum-immune pig. House C, which also contained 3 susceptible pigs and 1 serum-immune pig, was also connected to house A by a screened flyway, but the flyway was closed on one end with screen wire to prevent birds from flying across. Thus, house C served as an air-contact or environmental control.

Fifteen English sparrows were released in the connected houses A and B. They quickly adapted to the confining life, were observed making numerous trips across the flyway every day, and were seen eating and drinking alongside the pigs. The droppings of these birds were found in the feeders and waterers of both houses.

The experiment was allowed to continue for six weeks during which time the pigs' temperatures were taken weekly and observations were made daily. At the end of the six weeks, all unaffected pigs were challenged using a 10^{-4} dilution of the HCV used in the original exposure. The sparrows were caught, killed, examined, and their bodies (skin and feathers removed) were fed to susceptible pigs.

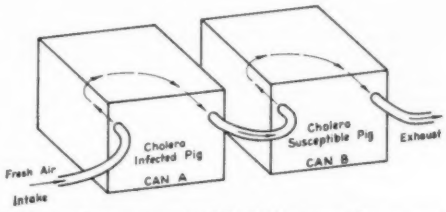

Fig. 1—Arrangement of isolation cans for aerosol transmission of hog cholera.

Trials 2 and 3.—These trials were conducted in a manner similar to trial 1 in most details; however, the air-contact control pigs were housed only 3 ft. away from the infected house A, while the bird contact control house B was still 6 ft. away. In addition, in trial 3, the bird-contact control pigs were each given, orally, 1.0 ml. of a 10^{-2} dilution of a 24-hour broth culture of *S. choleraesuis* one week before and again on the same day that the pigs in house A were inoculated with HCV.

Trials 2 and 3 were allowed to continue for eight weeks, at the end of which time all normal pigs were challenged by pen exposure with 1 of the cholera-sick pigs of house A.

Experiment 7—the Stablefly and Housefly.—Approximately 200 flies (stableflies and houseflies) were caught while they were feeding on the secretions and excretions of swine that were in the febrile stages of hog cholera. These hogs were part of a fast-spreading outbreak of cholera in northern Indiana. The validity of the field diagnosis of hog cholera was established by swine-inoculation trials, and the virus was designated as the Floyd Chapman strain.

The flies were placed in a ventilated mason jar and brought to the laboratory (25 miles away) where they were released in a small isolation room which contained 5 susceptible pigs. Body temperatures of the pigs were taken weekly, and observations were made daily for a total of 40 days. At the end of that time, surviving pigs were challenged by intramuscular injection with the Floyd Chapman strain of HCV.

Experiment 8—the Manure Worm.—Hog cholera-infected soil was prepared by digging earth from an area where there had been no manure piled and no animals kept; 500 ml. of fresh hog blood containing HCV was added and mixed throughout

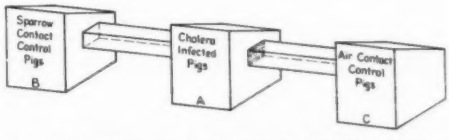

Fig. 2—Arrangement of portable isolation houses for English sparrow experiment.

the dirt. This was placed in a clean bucket and 100 ml. more of the virus was poured on the surface of the dirt so that the entire surface was soaked with virus-containing blood.

About 200 manure worms were placed on the surface of this infected soil, and within one hour they had burrowed below the surface. This method is similar to that used by Shope [29] to infect earthworms with lungworm larvae. The worms were allowed to remain for 48 hours in this bucket which was kept in an air-conditioned room (70–75 F.).

At the end of the exposure period, all viable worms were removed to a bucket of uncontaminated soil. After one day and again after five days in this soil, 30 worms were removed and washed in tap water until they appeared clean. They were then rinsed three times in Earle's balanced salt solution. The third rinse (20.0 ml.) was saved and the entire amount was injected into a cholera-susceptible pig as a control of the possibility that the virus might have been mechanically carried-over on contaminated soil. The worms were next ground with sterile sand in a mortar, and the emulsion was filtered through sterile gauze to remove any large particles. The resulting suspension was drawn into a syringe and injected into 3 susceptible pigs and 1 serum-immune pig.

These pigs were observed for 30 days. Temperatures were taken weekly and observations were made daily. At 30 days, the survivors were challenged by intramuscular inoculation of the virus material used to infect the soil.

EXPERIMENTAL RESULTS

PART I

Experiment 1—the Oral Route.—The virus control pigs of both trials developed typical symptoms, including leukopenia, and gross lesions commonly associated with hog cholera. Death occurred between nine and 13 days after the exposure to the virus. These results established that the virus used in the experiment possessed a titer of at least 10^6 infective doses per milliliter.

The *S. choleraesuis* control pigs developed a transient fever which lasted three days. They quickly returned to normal and showed no other ill effects. This indicated that the *S. choleraesuis* culture alone, in the dilution given, apparently had not induced serious disease. These pigs were susceptible to HCV when challenged intramuscularly at the end of the three-week observation period.

The untreated environmental control pigs remained unaffected during the three-week observation period even though pigs were dying of hog cholera in isolation cans adjacent to them. They were shown to be susceptible when challenged by intramuscular inoculation of HCV.

Results were irregular in both trials when HCV alone was administered by the oral route; 2 of 10 exposed pigs developed hog cholera. These results were reminiscent of the irregular results reported by some other workers [9, 15, 32] using this mode of exposure. Only the pig given the 10^{-2} dilution of HCV in the first trial and the pig given undiluted HCV in the second trial were affected by oral exposure. The 8 unaffected pigs did not develop fever, leukopenia, or any other evidence of disease during the three-week observation period. When these 8 pigs were challenged by intramuscular inoculation of HCV, all were found susceptible except the pig in the first trial that ingested 1.0 ml. of undiluted HCV without becoming sick.

In contrast to the above group, all 10 pigs exposed orally to both HCV and *S. choleraesuis* developed cholera. Some of these pigs exhibited a rise in body temperature 24 hours after the administration of the mixture, which was probably caused by *S. choleraesuis*, but the majority showed no ill effects from the exposure until the fifth or sixth day when body temperatures elevated to 104.0 to 106.5 F. At that time, they began to go off feed, and became weak, depressed, and incoordinated. Irregular-shaped purple blotches were common on the skin of these pigs. This lesion was common to pigs affected with the Carl Johnson strain of HCV.

The pigs that received the higher dilutions (10^{-3} through 10^{-6}) of the virus mixed with *S. choleraesuis* did not develop as acute an infection as did those given the lower dilutions. These more chronically affected pigs seemed to improve after the first two weeks of illness; however, they continued to lose weight, showed mild depression, and a few days before death became paralyzed and exhibited symptoms associated with encephalitis. At autopsy of these chronically affected pigs, lesions included button ulcers about the ileocecal valve and degenerative changes of the kidneys. To establish whether these pigs actually had hog cholera, a susceptible and a serum-immune pig were placed in the isolation can of each of these chronically affected pigs on the sixteenth day after the original exposure. The suceptible pigs developed acute hog cholera, while the serum-immune pigs were unaffected by the exposure, thus indicating that the disease present in the chronically affected pigs was hog cholera.

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White blood cell counts of the HCV-*S. choleraesuis* group varied from 3,500 to 10,000 cells per cubic millimeter, with the greater leukopenia occurring in those pigs that were more acutely affected.

Salmonella choleraesuis was cultured from the spleens and kidneys of all pigs in the HCV-*S. choleraesuis* group. In the group in which only HCV was given, *S. choleraesuis* was not found in any organs cultured; however, it is interesting to note that another species of Salmonella, *S. typhimurium*, was isolated from the pig of this group that contracted cholera from the ingestion of a 10^{-2} dilution of HCV. Thus, of a total of 20 pigs given HCV orally, cholera was contracted by only 1 pig in which some species of Salmonella was not isolated from the tissues on autopsy. This pig received 1.0 ml. of undiluted HCV (at least 10^6 infective doses) orally in the second trial of the experiment. In the first trial, the pig that received undiluted HCV without *S. choleraesuis* did not become sick, but did apparently become immune from the exposure.

Experiment 2—the Conjunctival Sac.—All pigs exposed to HCV by way of the conjunctival sac contracted the disease with and without the presence of *S. choleraesuis*. The disease appeared to be acute in its course, and all animals were dead by the fourteenth day. At necropsy, lesions were primarily petechial hemorrhages in all organs examined. White blood cell counts were all below 5,000 cells/cmm.

Experiment 3—Topical Exposure of Abraded Skin.—Both pigs exposed to HCV via abraded skin developed clinically observable cholera in six and seven days, and died in 12 and 14 days.

Experiment 4—Topical Exposure of Unbroken Skin.—Results of this experiment were somewhat similar to those of experiment 1 (oral route). All 3 pigs that had been given undiluted HCV plus *S. choleraesuis* on apparently unbroken skin developed cholera, while only 1 of 3 pigs that received undiluted HCV without *S. choleraesuis* developed cholera. The 2 unaffected pigs were found to be susceptible to a virus challenge injection given three weeks after the skin exposure.

PART II

Experiment 5—Aerosol Vection.—All 9 HCV-inoculated pigs in cans A, and the 9 pigs exposed to them by direct contact, developed cholera, while only 6 of the 9 pigs contracted cholera when exposed indirectly through air currents coming from the contaminated cans. The 3 pigs that did not develop the disease were susceptible to cholera when they were placed in direct contact with the sick pigs in the adjoining cans. Two of these 3 unaffected pigs were part of the trial in which *S. choleraesuis* was given to the inoculated pigs four days before the HCV inoculation, and the third unaffected pig was from the trials in which *S. choleraesuis* was not a factor.

Experiment 6—the English Sparrow.—Trial 1.—At the end of six weeks, 1 pig in the sparrow-contact house had a temperature of 106.5 F. and seemed to be off feed. The other pigs in the pen appeared normal. Five days later, the temperatures were elevated in the other 3 pigs. The serum-immune pig's temperature remained high for four days before it returned to normal. Clinical signs, white blood cell counts, and lesions of the affected pigs were typical of hog cholera. The serum-control pig completely recovered after an initial debility and was immune to a challenge injection 60 days later.

The pigs in the air-contact control house remained normal during the six-week period and were shown to be susceptible to cholera by an intramuscular challenge injection of HCV.

When the bodies of the sparrows were fed to pigs, cholera did not develop and no immunity was detectable.

Trials 2 and 3.—At the end of the fourth week of observation, 1 pig in the sparow-contact house (trial 2) appeared to be somewhat off feed and its temperature was 105.2 F. The following week, it was moribund, and all other pigs in this house began to show inappetence, depression, and fever. The serum-treated pig in this house developed a fever which lasted about five days, after which the pig recovered.

The pigs in the air-contact control house, and those in the sparrow-contact house (trial 3) all remained normal during the eight weeks of the experiment. All these pigs were susceptible to cholera when challenged by pen contact with a sick pig from one of the "infected" houses.

All pigs fed the bodies of the sparrows in trials 2 and 3 remained healthy during a three-week observation period and were found to be susceptible at the end of that

time by an intramuscular challenge inoculation of HCV.

Experiment 7—the Stablefly and Housefly.—All 5 pigs remained apparently healthy and vigorous during the 40 days of contact with flies caught while feeding on the secretions and excretions of hogs dying of hog cholera. At the end of the exposure period, these pigs were shown to be susceptible to cholera by intramuscular challenge with HCV.

Experiment 8—the Manure Worm.—All 4 pigs inoculated with the manure worm preparation remained apparently normal during the 30 days of observation. There was no elevation in body temperatures, and the sites of inoculation remained unaffected. On challenge, these pigs developed hog cholera. The serum-control pig lived 30 days before it died. This extended period was probably due to residual immunity from the serum inoculation 30 days previously.

DISCUSSION

The unpredictability of HCV to establish an infection when ingested by pigs was clearly shown in both trials of the oral route experiment. Only 2 of a total of 10 pigs exposed by this route became infected. It will be recalled that a number of investigators reported great difficulty in infecting hogs by the oral route.[9, 11, 15, 32]

In contrast to the above results, when HCV was given orally, along with an innocuous dose of *S. choleraesuis,* hog cholera developed in all 10 pigs exposed, even when doses of HCV as diluted as 1×10^{-6} were used. Thus, it would seem that *S. choleraesuis* is an important adjuvant factor in the transmission of hog cholera.

Further evidence that *S. choleraesuis* may have an adjuvant effect on the establishment of a hog cholera infection was presented by the experiment in which pigs were exposed to undiluted HCV on the apparently unbroken skin. One of 3 pigs given HCV in this way, without *S. choleraesuis,* contracted cholera, while all 3 pigs that were given both the virus and *S. choleraesuis* developed cholera.

The conjunctival sac and the abraded skin appeared to be routes through which HCV could gain entrance and cause infection. The disease resulting from exposure by these routes was acute; it compared well with the disease as seen after parenteral inoculation, and was effected with and without the presence of *S. choleraesuis.*

Hog cholera was transmitted from infected to healthy pigs in two of three trials using English sparrows as vectors, while the disease failed to spread to the pigs in houses in which sparrows were prevented from entering. No explanation is offered for the trial in which the disease was not transmitted by sparrows to pigs that had been given *S. choleraesuis* orally one week before and again on the same day that the pigs in the adjoining house A were injected with HCV.

The aerosol experiments indicated that HCV can become airborne and be carried for short distances to infect susceptible pigs. Transmission was successful in six of nine trials. It is interesting to note, however, that in the sparrow-transmission experiment, susceptible pigs were housed as near as 3 ft. from infected pigs without contracting the disease. This would tend to indicate that while air-current transmission is possible, it is probably not an important method of transmission of hog cholera.

The data gathered from the earthworm and fly experiments indicated that these animals, under the experimental conditions imposed, were not capable of transmitting hog cholera.

SUMMARY

1) When varying concentrations of hog cholera virus (HCV) were given to pigs by the oral route, the incidence of infection was relatively low; only two of ten trials were successful. However, when similar amounts of HCV were mixed with an innocuous amount of *Salmonella choleraesuis* and given by the oral route, hog cholera ensued in all ten trials.

2) Hog cholera virus plus *S. choleraesuis,* when placed on the unbroken skin of pigs, caused cholera in all 3 pigs exposed, while only 1 of 3 pigs developed hog cholera when exposed to HCV without *S. choleraesuis.*

3) All pigs exposed to HCV by way of the conjunctival sac or the abraded skin contracted the disease with and without the presence of *S. choleraesuis.*

4) Transmission was successful in six of nine aerosol transmission trials in which susceptible pigs were exposed to the exhaust air from isolation cans containing HCV-infected pigs.

5) English sparrows, when allowed to fly from hog cholera-affected pigs to susceptible pigs, transmitted the disease in 2 of 3 trials.

Pigs housed only 3 ft. away from the affected pigs, but which had no contact with the sparrows, failed to contract cholera.

6) Attempts to transmit cholera using earthworms, stableflies, and houseflies were unsuccessful.

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Enhancement of Infectivity of Hog Cholera Virus

KENNETH E. COLLINS, M.S.

Ames, Iowa

HOG CHOLERA VIRUS (HCV) preparations employed in research are complexes of organ homogenates, blood, or serum. It is usually assumed that titrations of these materials in susceptible swine give a measure of the quantity of HCV present. Potentially infectious (occult) virus may, however, be present but difficult to detect.[4]

Shope demonstrated that occult HCV can be carried by swine lungworm.[2,3] Occult HCV has also been demonstrated in ultraviolet-irradiated preparations.[1]

In a preliminary experiment by the present author,[1] a sample of ultraviolet-inactivated HCV* was found to be activated by heating at 40 C. for 90 minutes. This preparation was found to be infectious in 2 swine at the highest dilutions tested (10^{-3}).

The experiments reported in this paper were undertaken to further investigate the occurrence of occult as well as infectious HCV in serum from infected swine.

MATERIALS AND METHODS

Virus.—Blood containing HCV was collected from 2 swine nine days after inoculations with approximately 10 i.d.$_{50}$ of HCV III which was used in previous work.[1] The pooled blood was defibrinated and centrifuged. The resultant serum was filtered through Selas No. 10 and 02 filter candles, then tubed and stored at −15 C. for 15 months.

Normal Swine Serum.—Serum was obtained from normal, hog cholera-susceptible swine and processed in the same manner as the virus-containing serum.

Other Materials.—Water used in diluent solutions was de-ionized, then autoclaved at 15 lb. of pressure for 30 minutes. Phosphate buffer solutions, 0.1 M, were prepared with mono- and dibasic sodium phosphate.

Time and Temperature.—All serum samples were treated at either 25 C. or 40 C. for 90 minutes in a constant temperature bath.

Trypsin Treatment.—One-half per cent by weight of Difco 1:250 trypsin was used. The preparations were gently shaken for 5 minutes, then at 5 to 10 minute intervals during the remainder of the treatment period. A small portion of the trypsin did not dissolve in the 25 C. preparations at pH 5.5, but remained as a thin, powdery, surface layer. Dissolution was complete in the 40 C. samples during the initial five-minute period.

pH Treatment.—The pH of virus-containing serum was 8.4 to 8.7 following storage. The pH of individual samples was adjusted to 5.5 by equilibration at 0 C. under one atmosphere of carbon dioxide. The samples were then sealed. Such samples had a pH of 5.5 after heating at 25 or 40 C. for two hours. One pH 5.5-equilibrated sample stored at 4 C. for four months had a pH of 5.3 and was cloudy in apearance. This sample was shaken for 30 minutes in a nitrogen atmosphere and then adjusted to pH 8.5 by the addition of several drops of pH 8.7 phosphate buffer. The turbidity was decreased by this manipulation. This sample was later readjusted to pH 5.5 by treatment with carbon dioxide followed by the addition of dilute hydrochloric acid.

Phosphate buffer solutions of pH 5.5 or 8.5 were used to prepare 10^{-4} dilutions of HCV.

Dilution Procedure.—Immediately after treatment, serial tenfold dilutions of preparations to be titrated were made with 0.85 per cent sodium chloride solution and stored at 4 C. In no case was the storage of diluted material longer than three months.

Titrations.—One-milliliter quantities of each tenfold dilution titrated were inoculated into one axillary space of each of two 30- to 75-lb. test swine. In the case of certain preparations in which 1 ml. of the 10^{-4} dilution failed to infect, 10 ml. was subsequently inoculated into additional swine to approximate a 10^{-5} dilution. When 10-ml. amounts were given, the inoculum was divided between the two axillary spaces.

Swine and Swine Management.—Information about test swine management, and diagnostic criteria has been reported previously.[1]

RESULTS

The effects of four variables (temperature, pH, concentration, and presence or absence of trypsin) on the HCV preparation are summarized (tables 1, 2).

The reference titers of the HCV material were found to be 4.5 to 5.0* (experiments 1 and 9).

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* This preparation did not infect 2 test swine which were subsequently infected following inoculations with virulent HCV.

* Logarithms of swine i.d.$_{50}$ doses per milliliter. Titer differences of 1 log or less are not considered to be significant.

The results of the experiments involving these variables indicate that adjustment of undiluted HCV material to pH 5.5 results in a 10^3 to 10^4 increase in infectivity titer. No such increase occurred when the pH of a 10^{-4} dilution of similar material was adjusted to pH 5.5. A comparable 10^4 increase in titer occurred when undiluted HCV material was acted upon by trypsin at pH 8.5 at 40 C. No titer increase of this magnitude occurred when a 10^{-4} dilution of HCV material was acted upon by trypsin under comparable conditions. In fact, none of the variables investigated in this work produced significant titer increases in 10^{-4} dilutions of the HCV material.

TABLE 1—Infectivity Titers of a 10^{-4} Dilution of Hog Cholera Virus Material Following Various Treatments

Experiment No.	Trypsin (%)	pH	Temperature (C.)	Titer*
1	none	8.5	25	4.5
2	none	8.5	40	3.5**
3	none	5.5	25	4.5
4	none	5.5	40	4.5
5	0.5	8.5	25	4.5
6	0.5	8.5	40	6.0
7	0.5	5.5	25	3.5**
8	0.5	5.5	40	†2.5**

* Logarithm of i.d.$_{50}$ doses per milliliter of undiluted HCV material.

** Ten times the normal inoculum volume of 10^{-4} dilution was used as the 10^{-3} dilution.

† None of the surviving swine in this titration developed cholera until challenged.

Two experiments (not in tables) were performed to determine whether HCV material diluted with normal swine serum permitted titer increases similar to that of the undiluted preparation.

In one of these experiments, a 10^{-4} dilution of HCV material was made using normal swine serum as diluent. This preparation was treated with 0.5 per cent trypsin at pH 8.5 and 40 C. (same as for experiments 6 and 14). A titer of 5.5 was obtained, indicating that no significant increase in titer had occurred.

In the second experiment, a 1:1 dilution of the HCV material with normal swine serum was given this same treatment. The titer of this treated preparation was 6.3, relative to initial HCV content. Compared to the 6.0 titer in experiment 6 and the 9.0 titer in experiment 14, it would appear that the undiluted state of the HCV material was necessary for the occurrence of a large increase in titer. A comparison of the 6.3 titer obtained in this experiment with the normal titer of 4.5 to 5.0 indicates that a slight increase in titer may have occurred.

An additional experiment was performed to check for possible reversibility of titer following successive treatments. A sample of undiluted HCV material was treated at pH 5.5 and 25 C. (same as experiment 11). This sample was held at 4 C. for four months, after which the pH was 5.3. This preparation, after this four-month storage, was found to be infectious for 2 swine at 10^{-8} dilution. Three weeks later, the pH of this preparation was adjusted to 8.5 as previously described and maintained at 25 C. for 90 minutes, after which it was maintained at 4 C. for approximately 40 hours before inoculation. Dilutions of 10^{-6}, 10^{-7}, and 10^{-8} were inoculated into test swine. None of these swine developed hog cholera. All developed hog cholera following subsequent challenge. Readjustment of the pH of this sample to 5.5, followed by heating approximately two hours at 25 C., resulted in a preparation which was infectious for 2 swine at a dilution of 10^{-7}. No higher dilutions were tested.

TABLE 2—Infectivity Titers of Undiluted Hog Cholera Virus Material Following Various Treatments

Experiment No.	Trypsin (%)	pH	Temperature (C.)	Titer*
9	none	8.5	25	5.0
10	none	8.5	40	6.5
11	none	5.5	25	9.0
12	none	5.5	40	8.0
13	0.5	8.5	25	5.0
14	0.5	8.5	40	9.0
15	0.5	5.5	25	5.0
16	0.5	5.5	40	6.0

* Logarithm of i.d.$_{50}$ doses per milliliter of undiluted HCV material.

DISCUSSION

Increase in the infectivity titer of the HCV preparation may be attributed to an increase in the number of infectious virus particles or to an increase in the probability of initiation of infection by the virus particles. An increase in the number of infectious particles may occur due to dissociation of virus clusters or virus inhibiting complexes, or by assembly of infectious virus from noninfectious components.

The results obtained in this work are interpretable on the basis of an assembly of infectious from noninfectious components.

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Such an assembly is suggested by the marked dilution-inhibition of titer increases. Dissociation processes are equally consistent with the titer enhancements observed with undiluted HCV material. Simple dissociation mechanisms, however, do not appear adequate to explain the observed dilution-inhibition effect insofar as dilution is expected to favor dissociation processes, such as virus dissociation from inhibiting complexes or from virus clusters.

Furthermore, other possible processes, such as morphologic and chemical rearrangement of noninfectious virus to produce infectious virus, and the possible effect of components of the HCV preparation on the susceptibility of the host must be considered. The data obtained in this study are not adequate to establish the role of these mechanisms in the production of the observed HCV titer enhancement.

SUMMARY

Hog cholera virus (HCV) in blood serum, both diluted and undiluted, was subjected to different conditions of pH, temperature, and trypsin content. The treated preparations were then titrated in susceptible swine. No significant increases in infectivity titer were found for the diluted preparations. Large (ten thousand-fold) increases in the measured $i.d._{50}$ per milliliter were found for undiluted preparations treated at pH 8.5 and 40 C. in the presence of 0.5 per cent trypsin or at pH 5.5 and 25 C. in the absence of trypsin. In the latter case, the infectivity titer was found to be reversibly dependent on pH.

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The Passage of Hog Cholera Virus Through Cellulose Dialysis Membranes

KENNETH E. COLLINS, M.S.

Ames, Iowa

In 1927, Roderick and Schalk [6] reported the passage of hog cholera virus (HCV) through three different kinds of dialysis membranes: parchment, collodion, and Goldbeater's skin. It was found that HCV passed through these membranes into normal swine serum and normal horse serum but did not pass through the membranes into distilled water, NaCl solution, or Ringer's solution. In 1928, Kernkamp [3] also obtained evidence of the passage of HCV through collodion membranes.

Passage of HCV through dialysis membranes is unexpected since such membranes retain 5- to 8-mμ diameter molecules such as serum globulin, hemoglobin, and albumin while well-studied viruses have diameters greater than 8 mμ.[7] Passage of a small number of virus particles through abnormally large pores of membranes is possible, however, and this possibility has been considered by Bland [1] to account for the reported passage of neurovaccine particles through collodion membranes.[4] Nevertheless, the possibility exists that some infectious component of HCV preparations is sufficiently small to pass through membrane pores which do not pass molecules 5 to 8 mμ in diameter.

The present set of experiments was designed to determine whether an infectious HCV component is capable of passing through cellulose membranes.

Experimental Procedures

Virus and Virus Detection.—Swine serum containing HCV was the source of virus for these experiments. It was obtained from 2 hog cholera-infected swine nine days following inoculation with virulent HCV. The infectivity titer of this serum was approximately 10^5 swine infective doses per milliliter.[2]

Hog cholera virus was detected by inoculation into susceptible swine.

Dialysis Procedures.—Following overnight soaking of a 6-inch length of 20/32-inch diameter cellulose dialysis tubing * in water, a knot was tied near one end. Care was taken to prevent stretching of the resulting dialysis "bag." This bag was placed over the outside of a 1/2-inch (outer diameter) glass tube (fig. 1) and fastened by means of a rubber band. The glass tube and dialysis bag were lowered into an outer glass tube which contained 10 ml. of wash fluid (fluid No. 2, fig. 1). Fluid No. 1, 2.5 ml. of HCV, was then placed in the dialysis bag by means of a long needle. The glass rod stirring system stirred fluid 1 directly and, by vibration, agitated fluid 2. The dialysis system was maintained at 25 C. by means of a constant temperature water bath.

Following dialysis, the inner tube and dialysis bag were carefully taken from the outer glass tube. Fluid 2 was then transferred with a needle and syringe into a capped bottle.

Buffer Solutions and pH Adjustment.—For two experiments (6 and 7), a 15-ml. portion of the HCV serum was adjusted to pH 5.5 by means of slow dropwise addition of 1:10 hydrochloric acid, with steady stirring.

One tenth molar phosphate solutions at the desired pH were made by mixing 0.1 M monobasic sodium phosphate solution and 0.1 M dibasic sodium phosphate solution until the desired pH was obtained.

Results

Experiments 1 to 3 (table 1) involved dialyses of the HCV serum against normal swine serum. Experiments 1 and 2, employing membranes made from two different rolls of cellulose dialysis tubing, verified the observation of Roderick and Schalk [6] that HCV passes through dialysis membranes into normal swine serum. Experiment 3 indicated that the quantity of HCV passing into the normal swine serum in eight hours of dialysis was apparently less than several thousand infective doses.

Experiments 4 and 5 were performed with HCV against aqueous buffer solutions at pH 8.5 or 5.5. This was done to check the possibility that the nonpassage of HCV into water, NaCl solution, or Ringer's solution, in Roderick and Schalk's experiments, might have been due to iso-electric precipitation processes within the dialysis mem-

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* Visking "Nojax" dialysis casing, Visking Corp., Chicago, Ill.

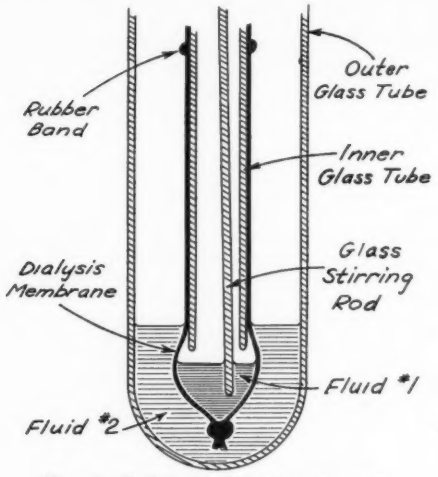

Fig. 1—Design of dialysis equipment.

branes as a result of a pH gradient across the membranes. The nondetection of HCV in the wash fluid (experiment 4) indicates that factors other than iso-electric precipitation may be involved. The pH 5.5 buffer experiment (experiment 5) was performed to determine if this pH, which has been found to increase the infectivity titer of the HCV serum,[2] would permit passage of HCV. The nondetection of HCV in this experiment could have been due to precipitation reactions within the membrane.

Experiments 6 and 7 were designed to determine if the HCV serum, adjusted to pH 5.5, would pass through cellulose membranes. The detection of HCV in a 1:200 dilution of the wash fluid indicates the passage of numerous infectious particles through the membrane.

DISCUSSION

The observed passage of HCV through cellulose membranes can be accounted for by the existence of an infectious HCV component which is smaller than 5 to 8 mμ. It is likewise possible to account for the passage of HCV through the membranes in terms of virus particles of typical size passing through a few relatively large pores in the membranes.

Renkin [5] has measured the average pore diameter of a commercial brand of cellulose membrane by several methods. Values of 1.5, 2.60, 3.0, and 3.78 mμ were obtained by the different methods. A value of 2.8 ± 1.4 mμ was given as indicative of the distribution of pore diameters.

The experiments reported here are of a preliminary nature, but they suggest that an infectious HCV component may be small compared to other viruses, perhaps smaller than 4 mμ in diameter. The possibility that a component of HCV of this size carries the necessary replication information warrants systematic investigation.

Dialysis techniques might be used to obtain HCV preparations of high purity. A pH 5.5 dialysis, for instance, might permit the separation of HCV from much of the contaminant protein material. A second dialysis at a higher pH might then be expected to free the HCV preparation from undesired salts.

TABLE 1—Dialysis of Hog Cholera Virus (HCV) Serum Preparations Against Various Fluids

Experiment	HCV preparation (fluid No. 1)	Wash fluid (fluid No. 2)	Dialysis time (hr.)	Test inoculum	Swine inoculated		
					Number	Positive	Negative*
1**	HCV serum, pH 8.5	Normal swine serum, pH 8.4	8	1 ml. of wash fluid	2	2	..
2	HCV serum, pH 8.5	Normal swine serum, pH 8.4	8	1 ml. of wash fluid	1	1	..
3	HCV serum, pH 8.5	Normal swine serum, pH 8.4	8	1 ml. of 1:200 dilution of exper. 2 wash fluid	2	..	2
4	HCV serum, pH 8.5	pH 8.5 buffer	8	1 ml. of wash fluid	2	..	2
5	HCV serum, pH 8.5	pH 5.5 buffer	8	1 ml. of wash fluid	2	..	2
6	HCV serum adjusted to pH 5.5	pH 5.5 buffer	8	1 ml. of wash fluid	2	2	..
7	HCV serum adjusted to pH 5.5	pH 5.5 buffer	8	1 ml. of 1:200 dilution of exper. 6 wash fluid	2	2†	..

* Susceptible to hog cholera when challenged.

** This experiment performed with membrane from a different roll of cellulose tubing than used in subsequent experiments.

† The first signs of hog cholera appeared 12 and 14 days after the inoculation. Both swine were moribund with hog cholera within six days of the first signs.

SUMMARY

Hog cholera virus in blood serum was dialyzed against normal swine serum and against phosphate buffer solutions, using cellulose dialysis membranes. Hog cholera virus was found to pass through the membranes into normal swine serum at pH 8.5 and into phosphate buffer solution at pH 5.5.

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A Relationship Between Vitamin B_{12} and Reproduction, and a Method of Diagnosing Vitamin B_{12} Deficiency in Individual Swine

G. L. FREDERICK, D.V.M., M.Sc.

Ottawa, Ontario

A HIGH INCIDENCE of sterility and of embryonal and neonatal mortality occurs in swine of various countries.[1,2,12,13,15,16] At the Central Experimental Farm in Ottawa, no significant genetic, toxic, infectious, or antigenic etiologic factors have been found; the diets conform to current nutritional standards.

Because the contemporary trend is to feed less animal protein and the information on the actual requirements of adult swine is insufficient to provide a factual basis for the current practices of vitamin B_{12} supplementation, it is possible that a vitamin B_{12} deficiency state, similar to that in vegans,* exists in swine. This could contribute to some of the reproductive problems of swine. Also, it might invalidate a general survey of serum vitamin B_{12} levels to determine the optimal range of swine and any attempt to correlate the serum levels of individual sows with their reproductive ability.

The physiologic response of sows to the intramuscular injection of a specific dose (250 μg.) of vitamin B_{12} has been reported.[4] It indicated that the serum vitamin B_{12} levels in individual sows, between 24 and 336 hours after the injection, might be their optimum levels, and also that the ratio of the preinjection to the postinjection serum levels within this period might indicate the vitamin B_{12} nutritional status of *individual* sows. It was mentioned that 3 of the sows were barren (*i.e.*, did not produce young) and that they had significantly lower ratios than 4 fertile sows, although there was no statistically significant difference in the preinjection serum levels.

The work reported here was undertaken to elaborate on the relationship between vitamin B_{12} and reproduction in the Ottawa swine herds and to investigate the reliability of using the blood serum vitamin B_{12} ratio as a means of assessing the vitamin B_{12} status of any individual sow with respect to reproductive ability as influenced by vitamin B_{12}.

EXPERIMENTAL PROCEDURE

Preliminary information on sows (No. 1 to 7) was included in the previous report.[4] The data were collected intermittently after two different, unrelated farrowing seasons, without regard to the fertility status of the individual sows. The status was determined later. Just prior to and during the individual gestation periods studied, these sows were fed a commercial sow diet that contained a vitamin B_{12} supplement in amounts recommended by the manufacturer. The total vitamin B_{12} content of the diet was approximately 4 to 5 μg./lb. (by both calculation and intermittent assays).

Sows A to R were studied following a different farrowing season. This time, the barren and fertile sows were selected at random from their respective groups within the herd. They had been fed a commercial sow diet without a vitamin B_{12} supplement during their gestation period. The approximate vitamin B_{12} content of this diet was 2μg/lb.

All results were pooled, regardless of previous dietary intake; the only discrimination used was whether or not the sows brought forth living young. A total of 12 fertile and 11 barren sows was tested.

The sows had been fed a farrowing ration prior to selection. After selection, they were fed the same diet as prior to and during the gestation period for at least ten weeks before the test was made. Previous work[5] indicated that this allowed sufficient time for the serum vitamin B_{12} levels of the sows to stabilize to their new dietary intake of vitamin B_{12}. It was assumed that the vitamin B_{12} nutritional status, as indicated by the blood serum ratio at the time of the test, was indicative of that before and during the gestation period. This assumption was necessary because of the difficulty of applying the test to a sufficiently large group of animals prior to or during their gestation periods before the fertility status of the individual animals was known, and because of the possible

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* A vegan is one who eats only foods of vegetable origin.

beneficial influence that the injected vitamin B_{12} might have had in the sows. The respective gestation diets were fed *ad libitum* during the tests. The sows were not fasted prior to bleeding.

Blood samples were taken from all sows immediately prior to the intramuscular injection of 250 μg. of vitamin B_{12} and at 24 and 48 hours after the injection. This specific dosage was used because it allowed the flooded postinjection serum vitamin B_{12} levels to fall to plateau levels within 24 hours; also, it maintained the plateau levels for at least another 312 hours, when sows 1 to 7 were fed the ration with the vitamin B_{12} supplement during the test period.[4] The two postinjection bleeding times were selected for reasons of convenience in performing the tests and to confirm whether or not the serum levels for sows A to R remained approximately constant during this time interval after the specified dose, despite their lower dietary intake of vitamin B_{12} during the test.

Blood serums were assayed by means of a modified technique (using *Euglena gracilis*) previously described.[6]

Shortly after the tests, the reproductive tracts were examined at slaughter to eliminate any sows with gross anatomic abnormalities or infections that might have interfered with fertility and might not have been related to a vitamin B_{12} deficiency. One barren sow was rejected because of infantile uterine horns; all other sows had macroscopically normal genitalia and were included in the study.

TABLE 1—Vitamin B_{12} Serum Levels ($\mu\mu$g./ml.) of Fertile and Barren Sows Before and After Intramuscular Injections of 250 μg. Vitamin B_{12}

Group	Sow	0 hr.	24 hr.	48 hr.	$\bar{x}$*	Ratio
Fertile	1	220	278	300	289	0.76
	2	332	432	452	442	0.75
	3	240	303	375	339	0.71
	4	188	287	306	297	0.63
	J	330	462	551	507	0.65
	K	308	348	367	358	0.86
	L	564	564	647	606	0.93
	M	438	523	484	504	0.87
	N	248	289	239	264	0.94
	P	493	817	787	802	0.61
	Q	359	397	456	427	0.84
	R	276	449	382	416	0.66
Mean		333			438	0.77
s.e. of mean		33.1			44.3	0.034
Barren	5	239	356	486	421	0.57
	6	109	206	250	228	0.48
	7	126	305	292	299	0.42
	A	438	802	761	782	0.56
	B	246	415	399	407	0.60
	C	351	460	384	422	0.83†
	E	255	458	415	437	0.58
	F	140	490	412	451	0.31
	G	199	449	304	377	0.53
	H	339	552	628	590	0.57
Mean		244			441	0.55
s.e. of mean		33.9			48.3	0.042

$\bar{x}$ = the mean of the 24- and 48-hour serum levels.
† See text for discussion of this outlying value.

RESULTS

The results are tabulated (table 1).

As in the previous work with the same dose of vitamin B_{12},[4] paired t-tests showed no significant differences between the 24- and 48-hour postinjection serum levels, thus indicating an approximately stable state. Therefore, the means of the 24- and 48-hour postinjection serum levels of individual sows were used in comparisons of the barren and fertile groups of animals.

The mean postinjection levels for the two groups of sows differed by only 3 $\mu\mu$g./ml. Although the preinjection levels for the fertile sows were 36 per cent greater than that for the barren sows, the variability from sow to sow within a group was relatively high, and the mean difference did not reach significance at the 5 per cent level. However, when the relationship between preinjection and postinjection levels was taken into account, the variability was much reduced, and a clearer discrimination between the two groups was possible.

Analysis of covariance indicated that the relationship between preinjection and postinjection levels of serum vitamin B_{12} for the groups could be represented adequately by straight lines through the origin, and the difference between the slopes of these two lines was significant ($P < 0.01$). Because the scatter about these lines also tended to increase with distance from the origin, the ratio of the two vitamin B_{12} serum levels was used to find a discriminant for the two groups, assuming a normal distribution for this ratio.

The value midway between the ratios for the two groups was 0.66. When this value was used as a discriminant level and animals with a lower ratio were classified as likely to be barren for reasons associated with vitamin B_{12} deficiency, the probability of misclassification for a single animal was approximately 19 per cent. A test for rejection of extreme values[3] supported the exclusion of the results for sow C, and it was reasonable that not all cases of sterility were associated with a vitamin B_{12} deficiency. Then the discriminant ratio was reduced only to 0.64, but the estimated probability of misclassification was reduced to about 13 per cent. From a practical point of view, the lower discriminant ratio (0.64) might be the better one to use, but the higher probability of misclassification with the higher discriminant (0.66) might be a better guide to the reliability of the method.

The high ratio of sow C is important in that it indicates that low ratios are not common to all types of sterility.

If only the 24-hour postinjection serum levels were used to calculate the ratios, the probability of misclassification of any individual was 17.5 per cent when sow C was included, and 12.5 per cent when excluded. The corresponding figures for the 48-hour levels were 25.0 per cent and 17.0 per cent. Although it appears that the ratio can be determined as effectively when only the 24-hour postinjection sample is used, the two postinjection samples are recommended.

DISCUSSION

Some of the barren sows had preinjection serum levels which, on the basis of previous observations, seemed high and would not have been considered indicative of a vitamin B_{12} deficiency. However, when the specific postinjection (plateau) serum levels of individual sows were considered, differentiation was possible. It appeared that there was a relationship between vitamin B_{12} and some of the reproductive failures in the herd, and that the ratio was a better discriminant of this relationship and of the vitamin B_{12} status of individual sows than the preinjection serum levels by themselves.

The possible physiologic significance of the postinjection plateaus and of the ratios was discussed previously.[4] In addition to giving a measure of the vitamin B_{12} status of individual sows, the ratio may also prove useful in determining the dietary and therapeutic requirements of swine. According to this work, sows in our herd should have ratios of at least 0.60 to have a 93 per cent chance of producing living offspring. Although neither the vitality of the offspring nor the health of the sows was correlated with the magnitude of the ratios, it might be best to strive for ratios as high as 1.0 to ensure adequate nutrition.

Although a cause-effect relationship is not claimed, such would not be surprising. There is inadequate factual information on the requirement of adult swine for vitamin B_{12}. Estimates of the requirement for growing swine vary and are based on growth rate, a questionable criterion of physiologic adequacy from which extrapolations should not be made to pregnant adults. Richardson *et al.*[14] reported that additions of 5 and 10 μg. of vitamin B_{12} per pound of basal diet (corn-soybean oil meal) did not give significantly greater gains than the basal diet by itself; however, 20 μg. per pound did ($P = 0.05$). Nesheim *et al.*[11] estimated that the oral requirement of the baby pig was 20.0 μg./kg. of dry matter consumed, or approximately 1.2 μg./kg. of body weight. If adult and growing swine have the same requirement, based on body weight or dietary intake, the current recommendation of 25 to 50 μg. per sow per day is inadequate. In addition, more sensitive biochemical criteria of nutritional adequacy usually indicate higher requirements than growth rate studies. At present, the optimum criterion to which requirement can be related is unknown for all species. It is known that various dietary constituents do influence the requirements of rats, mice, chickens, and swine for maximum growth.

The influence of vitamin B_{12} on fetal development in swine was demonstrated by Johnsen *et al.*[7] Sows fed an all-plant diet without supplementary vitamin B_{12} ovulated 12.3 ova; sows fed the same diet plus 80 μg. of vitamin B_{12} orally per day, 11.7 ova. Four weeks after breeding, there were 5.6 embryos per sow in the first group and 10.4 embryos per sow in the second. Since nearly a 100 per cent fertilization rate may be assumed for the ova shed by fertile sows and other polytocous animals,[10] the degree of vitamin B_{12} deficiency present in the above sows caused approximately 50 per cent embryonic mortality even within the first four weeks of gestation. It is not unreasonable that a greater degree of deficiency could cause 100 per cent mortality within the same or longer period or, possibly, even a failure to conceive.

Successive pregnancies do accentuate the effects of a vitamin B_{12} deficiency in rats, and barrenness may occur.[8] The effect of successive pregnancies has been observed in this laboratory in sows fed a semisynthetic, vitamin B_{12}-deficient diet (to be reported). Fairly frequent menstrual disturbances associated with low serum vitamin B_{12} levels, but without megaloblastosis, have been reported in female vegans.[17]

The physiologic basis for the ratio exists in man,[9] and it probably exists in other animal species.

SUMMARY

Twelve fertile and 10 barren sows were given 250 μg. of vitamin B_{12} intramuscularly. Blood samples were taken prior

to the injections and 24- and 48-hours after the injections. Serums were assayed for vitamin B_{12}. It appeared that there was a relationship between vitamin B_{12} and the reproductive failures studied, and that the ratio of the preinjection to the postinjection serum levels was a better discriminator of this relationship and of the vitamin B_{12} status of *individual* sows than the preinjection serum levels by themselves. Discriminant analysis revealed that the probability of an incorrect classification may be 13 to 19 per cent when the ratio is used to detect individual animals barren for reasons associated with a vitamin B_{12} deficiency. Sows in these herds should have ratios of 0.60 or greater to have at least a 93 per cent chance of producing living offspring. The possibility of a cause-effect relationship is discussed.

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Immunization Against Mycoplasma Infections of Poultry

H. E. ADLER, D.V.M., Ph.D.; DUNCAN McMARTIN, B.V.M.S., M.R.C.V.S.; MOSHE SHIFRINE, Ph.D.

Davis, California

IMMUNITY to *Mycoplasma* following recovery from natural disease has been shown in bovine, caprine, ovine, and murine species. Cattle can be immunized with live cultures attenuated by propagation in artificial mediums [6] and embryonating chicken eggs.[5] *In vitro* propagation of the goat strain did not reduce virulence sufficiently for the organism to be used as a vaccine for goats and sheep.[2] Formalin-inactivated products, however, seemed to protect these species.[4] A heat-killed suspension of Mycoplasma protected the majority of test mice against the mouse Type B organism.[7]

Information on the immune response of chickens and turkeys is scant. Van Roekel and Olesiuk [8] reported that resistance to challenge was increased in birds recovered from natural disease. Adler [1] failed to protect turkeys with various killed vaccines or with cultures propagated through 90 passages in broth.

MATERIALS AND METHODS

Mycoplasma Strains.—All strains are designated herein by a capital letter; the letter "g" indicates that the figure following is the number of *in vitro* passages. The pathogenic S_6 strain was used in most experiments on vaccine production and challenge. The F strain was used to evaluate the resistance of recovered chickens to the effect of Mycoplasma on egg production. The characteristics of the S_6 and F strains are described in another report.[9] All other strains used in contact experiments and from birds infected under field conditions were of the S_6 type. Previously lyophilized cultures were passaged twice in broth to obtain maximum growth of each strain before use.

Culture Mediums.—The mediums for this study were PPLO broth or agar (Difco) enriched with 1 per cent yeast hydrolysate and 10 per cent horse serum. Phenol red and 1 per cent glucose were added to the broth to determine growth by acid production. Glucose, as a readily available carbon source, was initially added to the medium used for vaccine production. It was later omitted when maximum growth was observed to lower pH drastically, with concomitant toxicity for the Mycoplasma.

Titration of Cultures.—Tenfold serial dilutions were made in the enriched phenol red broth with dextrose substrate. The diluted organism suspension, in volumes of 0.01 ml., was seeded on an enriched PPLO agar. Approximate end point of growth was determined from acid formation in the broth and from Mycoplasma colonies on the agar (at the various dilutions). The cultures used for challenge, infection, and immunization were diluted in tryptose broth.

Live and Killed Vaccine Preparation.—The 66th culture passage of the S_6 strain was concentrated by centrifugation for 15 minutes at about 12,500 g, and washed twice in buffered sodium chloride. The original culture contained about 10^9 organisms per milliliter, and the final product for vaccination, 2.5×10^9 organisms per milliliter. A killed vaccine was obtained by exposing a portion of the culture to ultrasonic vibrations (sonicating) for 30 minutes.*

A second killed product was prepared from the 53rd culture passage of the S_6 strain. The culture was centrifuged and sonicated as described previously, and the disrupted organisms were added to the supernatant fluid. Formalin (0.5%) and 10 per cent aluminium hydroxide** were added.

All other vaccines were whole live cultures, usually the 51st culture passage of S_6, containing about 10^9 organisms per milliliter.

Immunity Challenge.—In most instances, a culture of the S_6 strain from lyophilized seed was activated by two passages in serum broth. By the third day of incubation, there were about 10^9 organisms per milliliter in the second-passage broth culture. Portions of this culture were used either diluted or as whole culture. The birds were inoculated on the left side at a point 1 cm. below the anterior extremity of the pubic bone, to a depth of 2 cm. within the abdominal cavity. Two weeks later, the birds were necropsied for determination of the extent of any air sac lesions. Lesions were classified as follows: grade 1, not over 2 to 3 mm., and well confined to the point of inoculation; grade 2, extending from the site of inoculation but not

From the School of Veterinary Medicine, University of California, Davis.

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* Raytheon Sonic Oscillator, Model DF 101, 250 watt 10 kc., manufactured by Raytheon Manufacturing Co., Waltham, Mass.

** Amphojel, produced by Wyeth Laboratories, Inc., Philadelphia, Pa.

to adjacent air sacs; grade 3, purulent exudate extending from the inoculated air sac to others.

Experimental Chickens and Turkeys.—The chickens and turkeys were obtained from flocks free of chronic respiratory disease. All birds were first tested for Mycoplasma agglutinins by the serum plate method.

RESULTS

Titration of Fifty Per Cent Infectious Dose.—Each culture dilution was inoculated in the abdominal air sacs of 5 Broad Breasted Bronze turkeys, 36 days old, and 8 or 10 Leghorn chickens 42 days old. The undiluted culture (S_6g5) contained 8×10^8 organisms per milliliter. The birds were necropsied two weeks after inoculation and the fifty per cent infectious dose ($i.d._{50}$) was determined on the basis of air sac lesions. The $i.d._{50}$ of this culture was $10^{7.4}$ in turkeys and $10^{6.5}$ in chickens. Lesions and agglutinins were positively related only in birds infected with 10^5 organisms per milliliter or more.

Spreading Potential of Vaccine.—Ten 8-week-old turkeys were inoculated i.m. with S_6 of the 23rd passage, containing about 10^9 organisms per milliliter. Two turkeys showed respiratory disease within two weeks. Twenty 6-week-old turkeys were inoculated i.m. with S_6 of the 50th passage, and an equal number was placed in the unit as contact controls. Two months later, at necropsy, the contact birds were free of disease and of serum agglutinins for the S_6 strain. In all of the following experiments, contact controls were used; none became infected. Intraperitoneal inoculation of the S_6 strain of the 50th passage produced aerosacculitis in turkeys and chickens of various ages.

Titration of Minimum Immunizing Dose.—Twelve to 15 White Leghorn cockerels, 7 weeks old, were given each log dilution of vaccine. Each milliliter of culture contained 6×10^8 organisms. One month after vaccination, the immunity of the birds was challenged with about 10^6 organisms. Protection was complete with about 10^7 organisms, and not significant with about 10^6 Mycoplasma.

Immunity as a Result of Recovery from Disease.—Fourteen adult Broad Breasted Bronze turkeys that had been diseased at about 3 months of age, and 14 Mycoplasma-free controls, were each allotted to two groups when 11.5 months old. The immunity of recovered birds in one group and of controls in one group was challenged with 10^9 organisms, both in the infraorbital sinus and in the abdominal air sac. The other groups were contact controls for the experiment. Six weeks later, all of the birds were necropsied. The mean grade of air sac lesions in recovered birds was 1.0 from inoculation and 0.6 from contact infection. In the controls it was 2.6 from inoculation and 1.3 from contact infection. Two birds in each of the groups of inoculated birds had sinusitis.

The Protective Effect of Recovery from Disease Against a Drop in Egg Production.—Ten 5-month-old Mycoplasma-free New Hampshire Red pullets were inoculated over the ovules with 1 ml. of S_6g18 containing 10^8 organisms. Based on weekly averages, egg production dropped about 16 per cent two weeks after exposure; full-sister controls showed no drop in production. Four months later the birds were exposed to 10^9 organisms of Fg20. Egg production dropped 12 per cent in the controls but remained unaffected in the birds that had had the disease.

Immunity Induced by Killed and Live Vaccines.—In the first experiment, groups of 8 cockerels, 7 weeks old, were inoculated with sonicated or live organisms by the intravenous, intramuscular, or intrabursal route. The dose was 1.0 ml. by either of the first two routes, and about 0.1 ml. by the last route. In agglutination tests three weeks later, titers were highest in serums from birds that were given the sonicated culture intravenously; next highest were from birds given live culture intramuscularly or intravenously. Two weeks after the agglutination tests, the immunity of the birds was challenged by injecting 10^6 organisms of S_6g7 into the abdominal air sac. Necropsy was performed two weeks later. The mean grades of air sac lesions (table 1) were: from sonicated culture—intravenous, 2.4; intrabursal, 2.25; and intramuscular, 1.25; from live organisms—intravenous, 0.75; intrabursal, 1.5; and intramuscular, 0.75. The mean grade of the control birds was 2.0. The highest grade, 2.4, was in the birds that had the highest serum-agglutinating titer.

The second bacterin was tested in a mixed group of Broad Breasted Bronze and Beltsville White turkeys, 4 months old. Sixteen turkeys were each given 1 ml. of bacterin (10^9 organisms). Eighteen birds were inoc-

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ulated i.m. with two doses of bacterin at a nine-day interval. Nineteen birds were given one dose of bacterin followed in nine days by 1 ml. of live S_6g51. Twenty-two turkeys were each inoculated with one dose of vaccine. Twenty-three turkeys served as unvaccinated contact controls. Ten days after the last immunizing dose, birds of all groups were placed in one large outdoor pen with 32 naturally infected turkeys of about the same age. Four months later, the turkeys were necropsied. The mean grades of air sac lesions were 0.93 from one dose of bacterin, 0.84 from two doses of bacterin, 0.71 from culture alone, and 0.62 from bacterin plus culture. The mean grade of the controls was 1.45.

TABLE 1—Serologic Response to Challenge of Chickens Vaccinated with Live or Killed Mycoplasma

Sonicated or live Sg66*	Route of inoculation	Mean agglutination titer**	Air sac lesion score following challenge
Sonicated	Intravenous	1:36.0	2.40
Live		1:17.5	0.75
Sonicated	Intramuscular	1:1.5	1.25
Live		1:10.5	0.75
Sonicated	Intrabursal	1:1.0	2.25
Live		1:5.0	1.50
Control	.....	0	2.0

* Eight chickens were used per group.
** Agglutination tests were conducted three weeks after immunization.

Immunization with Culture.—Twelve 9-week-old turkeys were inoculated i.m. with 10^9 organisms of S_6g51; 12 others served as controls. Three weeks later the immunity of the birds was challenged with 10^9 organisms. The mean air sac lesion grade was 1.4 in the vaccinated group and 3.0 in the controls.

Fourteen 8-week-old chickens were similarly immunized and challenged. The mean air sac lesion grade was 0.0 in the immunized group and 2.0 in the controls.

Duration of Immunity.—Forty-four 6-week-old chickens and 50 poults, 4 weeks old, were inoculated i.m. with one dose of S_6g50 containing 10^9 organisms. Six weeks after attempted immunization, the immunity of 25 of the poults was challenged with 10^6 organisms. The mean air sac lesion grade was 1.0 for the vaccinated turkeys and 1.7 for the controls. No further studies of duration of immunity were considered worthwhile in this group of turkeys.

In previous experiments, chickens had been found to be immune to challenge one month after inoculation. In this group, the first challenge with 10^9 organisms was two months after immunization. The air sac lesion grade was 0.2 in the vaccinated birds and 2.6 in the control birds. A second challenge of another group of birds was after three months, with 10^6 organisms. Mean grades of air sac lesions were 1.3 in the vaccinated birds and 2.1 in the control group.

DISCUSSION

The titration experiment to determine the $i.d._{50}$ showed that the turkey was far more susceptible than the chicken to Mycoplasma infection. This is not surprising, since epornities in the field, if uncomplicated, have always been more severe in turkeys than in chickens. In other experiments, the immune response to Mycoplasma was much poorer in turkeys than in chickens. If inoculated i.m., an attenuated culture apparently does not spread to the respiratory tract. This would be an important consideration in the production of a reliable immunizing product.

About 10^7 organisms appear necessary for immunization of chickens against Mycoplasma infection. Gray and Turner [3] made a similar observation in studies of immunization against bovine pleuropneumonia. The large number of organisms required and the great reduction in viability from lyophilization increase the difficulties involved in vaccine production.

Recovery from the disease apparently leaves some degree of immunity. Recovered turkeys showed about 50 per cent protection against challenge with large numbers of organisms. In the field also, it appears that breeder flocks that have had the disease very early in life do not readily shed the organisms in the egg and are less likely to be reinfected. In chickens, immunity induced by the disease seems to protect against the effect of Mycoplasma challenge on egg production. Unfortunately, Mycoplasma organisms were not recovered by our culture methods following ovule exposure of immune and control birds, though the rate of egg transmission was high in the pullets infected initially.

Preparations containing organisms killed by sonication or by formalinization did not protect chickens or turkeys against either inoculation or contact infection. Given in-

travenously, the sonicated organisms elicited a substantial agglutinin response, but these birds were the most susceptible to challenge. Live culture via the same route produced both antibody response and immunity to challenge. Studies are in progress to determine the relationship between agglutinins and immunity to challenge.

Washed cultures of S_6g66 gave poorer protection than was obtained later with whole cultures of earlier passages of the organism. Experience in this laboratory has shown that the method of handling the organism greatly influences its viability. Mortality is high if the organism is washed in buffer without taking great care to maintain correct osmotic pressures. Much of this information was not available when the experiment with the 66th culture passage was conducted. Thus, there could have been some reduction in the number of viable organisms present.

The large contact experiment with killed and live cultures gave results similar to those in the inoculation trials. When the time and cost of evaluating immunity are considered, direct inoculation may be as valid as contact exposure.

In the remaining experiments, the turkey did not react well to an immunizing dose of Mycoplasma; protection was only 50 per cent after three weeks and apparently very slight after six weeks. On the other hand, the chicken had good immunity for two months, though only fair immunity at the third month.

These experiments indicate that considerable work must be done before a suitable immunizing product against Mycoplasma can be obtained. The organisms must be protected against adverse osmotic and enzymatic effects, and adjuvants may be necessary.

Until the many difficulties are resolved, a conservative attitude should be adopted toward immunization against this infection with a vaccine. The study of the immune response of chickens and turkeys to Mycoplasma infection is worthy of continued effort to help solve the problem of controlling the infection, though it need not be directed toward vaccine production.

Summary

Observations were made on methods of inducing and evaluating immunity against Mycoplasma infection in chickens and turkeys.

Recovery from the disease confers measurable immunity in turkeys and chickens. A vaccine prepared by sonic disruption of Mycoplasma did not produce immunity in chickens. Similarly, an aluminum hydroxide-adsorbed, formalin-inactivated vaccine did not immunize turkeys. An attenuated culture administered intramuscularly immunized chickens for about two months; turkeys did not respond as well. Major technical difficulties were involved in preparing and maintaining the large numbers of viable organisms required for immunization.

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Characteristics of an Ornithosis Virus Strain of Low Virulence in Turkeys

CHARLES GALE, D.V.M., M.P.H., Ph.D.

Wooster, Ohio

ORNITHOSIS VIRUS was first isolated from turkeys in the United States by Boney *et al.*,[2] although it was thought to have occurred prior to this time.[11] Since the original isolation, the virus has been recovered by other workers.[1,4,6,12,14,16] Most of these strains have been highly virulent or toxic. In addition to these virulent strains, strains of low virulence have also been recovered from turkeys in epizootic areas as well as in areas where no epizooties have been reported.[9,10,16] Information regarding these strains should furnish valuable epizootiologic information. This report concerns the findings from studies of an avirulent strain of ornithosis virus in turkeys.

MATERIALS AND METHODS

The virus* used in this study was obtained from turkey tissues being screened for the infectious sinusitis agent; these tissues were obtained from a poultry plant at the time of processing.

All turkeys used in this report were obtained at 1 day of age from the station flock and held in isolation until used. Blood samples were obtained prior to exposure and the serum tested for antibodies against pleuropneumonia-like organisms by the hemagglutination-inhibition test as outlined by Fahey.[7] These serums were also tested prior to exposure for ornithosis antibodies by the direct complement-fixation and the indirect complement-fixation test.[3] Following exposure to the virus, blood samples were obtained at different intervals to determine antibody titers. In most instances, birds were checked at 30 days' postinoculation to determine if serologic evidence of infection was present. The titer levels were checked in several groups at 30, 60, 90, and 120 days.

The inoculum used to transmit the virus was infected yolk sac material and macerated third-passage mouse tissues consisting of the spleen and liver diluted to a 10 per cent suspension in phosphate buffer. The turkeys were allotted to various groups and exposed in groups by the following methods: intratracheally, intraperitoneally, intranasally, and contact. Groups of exposed and control turkeys were placed in isolation units. Body temperatures were taken twice daily.

The heart, liver, and spleen were obtained from exposed turkeys. These tissues were macerated, diluted to a 10 per cent suspension in phosphate buffer, and injected intraperitoneally into mice 3 to 4 weeks old. Bacteriologic examinations were made of the suspensions used to inoculate mice. On the tenth day, all surviving mice were killed and examined for lesions of ornithosis.

Leukocyte counts were made on turkeys at the time they were killed for the virus isolation procedure and from the remainder of the birds; blood samples were obtained from 6 exposed turkeys prior to killing them for tissues for virus isolation attempts during the first three weeks of exposure. Thereafter, blood samples were obtained from 3 exposed birds at regular intervals for the remainder of the exposure. Blood samples were obtained at each time from 2 controls killed at the same time throughout the exposure. The method of Wiseman, as modified by Olsen[15] (1935), was used for determining leukocyte counts. The diluting fluid consisted of 50 mg. of phloxine, 5 ml. of neutral formalin and 95 ml. of Ringer's solution. An erythrocyte pipette was used for diluting the fluid 200 times. The filled pipettes were allowed to stand at room temperature three to four hours prior to counting. Differential counts and morphologic studies were made from blood smears stained with Wright's stain.

RESULTS

Intraperitoneal Inoculation.—The virus was recovered from all birds inoculated intraperitoneally with the third-passage mouse tissues 72 hours following inoculation. Birds were held for various periods up to 180 days. The virus was recovered from 1 of 4 birds at 89 days and it was still present at 120 days but not thereafter. The titers and virus recoveries are summarized (table 1). Of the 31 turkeys, each 6 weeks old, none appeared sick at any time following injection.

Two more groups of 35 turkeys, each 6 weeks of age, were inoculated intraperitoneally. The results were similar to those of the first trial. Temperature reactions for this group are shown (fig. 1).

Intratracheal Inoculations.—Six-week-old turkeys in two separate pens of 70 were

Experimental data contained in this paper were obtained at the Ohio Agricultural Experiment Station, Wooster, and submitted to the Graduate School, University of Minnesota, Minneapolis, as partial fulfillment for the requirements of the Ph.D. degree, 1958.

* Virus furnished through courtesy of Dr. Pomeroy, University of Minnesota, St. Paul.

inoculated intratracheally. Those in one pen were inoculated with third-passage mouse tissue; those in the other, with infected-yolk material. The dosage was 0.25 ml. Samples were obtained from 20 birds in each group at 28 and 60 days. The titer, determined by the direct complement-fixation tests, varied in both instances from 0 to 128. The virus was recovered from 5 of the birds killed at 28 days. The virus was not isolated from 10 birds killed at 60 days. Temperatures of turkeys inoculated intratracheally are recorded (fig. 1). No losses or clinical signs of disease were found; no differences could be seen between the birds in these pens and the controls.

Intranasal Inoculation.—The 6-week-old turkeys were allotted to two groups of 30, and 0.5 ml. of third-passage mouse tissue inoculated into each sinus. Blood samples were obtained from these turkeys at 28 days following exposure; titers were 1:8 to 1:64. No signs of infection were observed 45 days following inoculation.

Contact Exposure.—Three groups of 10 unexposed turkeys, 6 weeks of age, were placed in three pens. Each pen already contained 65 turkeys exposed intratracheally at the time the contact birds were put into the pen. The unexposed birds failed to develop any signs of sickness. The virus was not recovered until 28 days after exposure to pen mates. Antibody titers did not develop until 37 days later, but these titers were lower (0 through 1:32) and tended to disappear in 28 days.

Virus Isolation.—The three groups of 65 turkeys used in the contact-exposure ex-

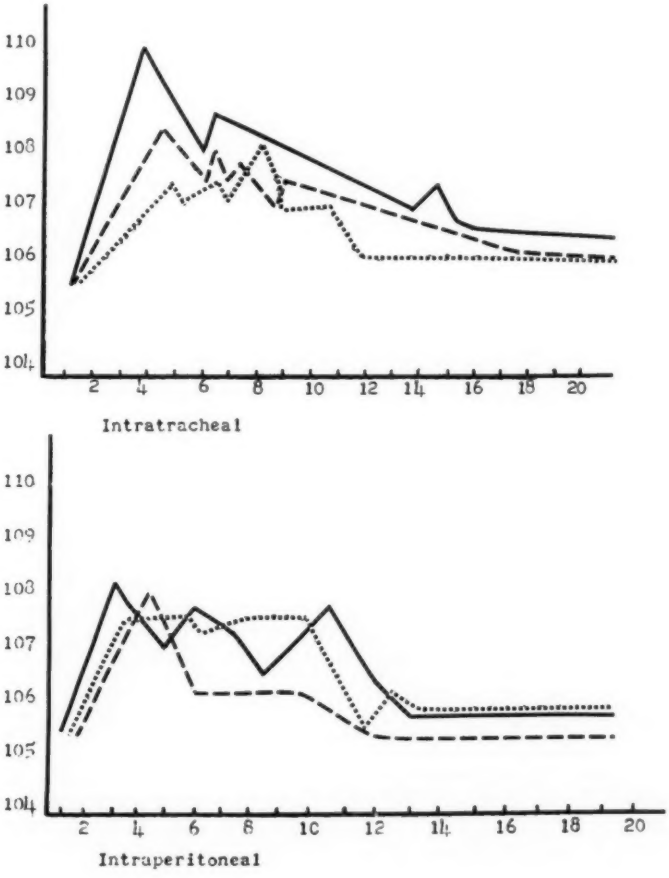

Fig. 1—Temperature reactions of several turkeys inoculated with the virus. Upper chart is for turkeys inoculated intratracheally and lower chart is for turkeys inoculated intraperitoneally.

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TABLE 1—Titers and Virus Recovery Following Experimental Ornithosis in Turkeys Exposed by the Intraperitoneal Route

Turkey (No.)	Reciprocal titer (DCF) direct complement-fixation: Days after inoculation 0	30	60	89	Agent isolated	Postinoculation day killed	Necropsy findings
829*	0	—	—	—	+	3	+
830	0	1/64	1/64	.....	...	....	...
831	0	1/32	1/8	.....	...	....	...
832	0	1/128	1/128	.....	...	....	...
833	0	1/64	1/64	.....	...	....	...
836	0	1/64	1/64	.....	...	....	...
837	0	1/64	1/32	1/8	—	89	—
838	0	1/32	1/8	.....	...	....	...
839	0	ac**	0	.....	...	....	...
840	0	1/64	1/64	1/8	+	120	—
841*	0	—	—	.....	+	9	+
842	0	ac	1/64	Neg.	—	89	—
843*	0	—	—	.....	+	9	+
844*	0	—	—	.....	+	10	+
845	0	1/128	1/8	1/128	+	89	—
846	0	—	—	—	+	4	+
847*	0	—	—	.....	+	10	+
848	0	1/128	1/32	Neg.	—	89	—
849	0	1/32	1/8	.....	...	....	...
850	0	1/32	1/16	.....	...	....	...
851	0	—	—	.....	+	19	+
852	0	0	0	.....	...	....	...
853	0	1/8	ac	.....	...	....	...
854	0	1/8	ac	.....	...	....	...
856*	0	—	—	.....	+	11	+
857	0	1/16	ac	.....	...	....	...
858	0	0	1/8	.....	...	....	...
859*	0	—	—	.....	+	11	+
860*	0	—	—	.....	+	12	+
834*	0	+	—	.....	+	12	—
835*	0	—	—	.....	+	15	+

* Killed prior to 30 days; ** ac = anticomplimentary sample.

periment were killed at different intervals for virus recovery studies (table 2). The virus was first isolated 72 hours following inoculation and could be recovered as late as 120 days. The stages of the disease were set up arbitrarily upon examination of the lesions. The virus could be readily isolated for 17 days and then the recovery rate diminished.

Age Susceptibility.—One-day-old poults, in three groups of 50 inoculated intratracheally, had a mortality of 36, 38, and 42 per cent. Mortality was recorded in all groups on the fourth day and continued through the tenth. Direct complement-fixation titers of the remaining poults at 28 days were found to be low, 0 through 1:16. Virus isolations were made from most of the poults which died. Of two groups of 30 poults, one group 3 days of age and the other 5 days of age had mortalities of 30 and 34 per cent, respectively. Ten-day-old poults in another group of 50 were found to have 2 per cent mortality, while in another group, 14 days old, no mortalities occurred.

TABLE 2—Stages of Experimental Ornithosis in Turkeys (Intratracheal Inoculation)

	Stage of disease	Time after inoculation (days)	Common lesions	Virus * isolation
1	Incubation	1–3	None	0/11
2	Acute	3–17	Pericarditis Periphatitis Aerosacculitis Pneumonia	65/65
3	Chronic	17–45	Chronic pericardial Adhesion, moderate Aerosacculitis	62/93
4	Carrier	45–120	Slight aerosacculitis Few chronic pericardial adhesions	4/21

* Numerator indicates number of positive isolates; denominator indicates number of turkeys examined.

Leukocyte Counts.—A rise in total leukocyte counts appeared to coincide with the temperature rise of inoculated birds (fig 1; table 3). Leukocytosis appeared to reach its peak at seven to eight days and then began to decline; it had returned to control range by the third week.

DISCUSSION

This strain of ornithosis virus caused a subclinical disease in turkeys above 2 weeks of age. Poults under 2 weeks showed heavy

mortality. These findings would tend to indicate that the susceptibility to the virus decreased with age, similar to observations made by Pate *et al.*[17] in natural and experimental cases of ornithosis. Three days after inoculation, body temperature rose 0.5 to 4.5 degrees and did not return to the preinfection range until 12 to 14 days later. In spite of this elevation of temperature, the birds did not at any time appear sick and there were no death losses.

An interesting observation was the rise in total leukocyte counts which appeared to coincide with the temperature rise. Meyer[13] states that leukopenia in man is present in only 25 per cent of the cases and that leukocytosis occurs in early convalescence. No reports were available on the blood picture of turkeys infected with ornithosis. Perhaps a larger sampling than made here might reveal other changes.

A serologic response, detected with the indirect and direct complement-fixation test, seemed to reach a peak approximately 30 days following inoculation and then decline. Turkeys under 6 weeks of age were less able to produce a high serologic titer response than birds 6 weeks or older. This was also noticed in preliminary observations with young poults. Wolfe[18] found that the ability of the chicken to produce antibodies increased after 5 weeks of age.

The virus injected intratracheally was found to persist in high concentration for short periods and was recovered in a high percentage of birds up to 17 days and was still present at 120 days. This is in contrast to Page's[16] work with the virulent New Jersey virus with a range of seven days.

Ornithosis viruses of low virulence have been reported previously, but the part that such viruses play in the epizootiologic picture of ornithosis has not been established. Such viruses may be more widespread than is currently known and are worthy of consideration.

Davis *et al.*[5] compared the effects produced by nine strains of ornithosis virus in turkeys and classified them into two groups: mildly pathogenic and highly pathogenic. They also state that older turkeys are more resistant to infection than young poults. It would be valuable to know the incidence of infection present in our turkey population. This would give some idea of the extent that subclinical infections might play in the epizootiologic picture of this disease. Page's[16] hypothesis that increased toxicity of the virus results in greater pathogenicity presents an interesting thought regarding this agent; the work here would tend to support this. While the effects of the viruses of low virulence appear to be stable, virulence is regarded as a matter of degree, and while this virus is of low virulence for adult turkeys, it does cause heavy losses in young poults and has resulted in human illness[9] from contact with this virus or a similar one.

TABLE 3—Summary of the Average Leukocyte Counts of Ornithosis-Inoculated Turkeys Taken at Various Times After Exposure

Days after exposure	Control turkeys	No. of birds	Inoculated turkeys	No. of birds
1	37,575	2	32,420	6
2	48,183	2	53,437	6
3	33,496	2	71,567	6
4	50,560	2	49,950	6
5	39,187	2	165,565	6
8	35,982	2	185,300	6
9	28,706	2	93,720	6
10	44,215	2	63,045	6
11	66,616	2	76,983	6
14	36,642	2	78,961	6
21	33,047	2	41,875	6
26	49,980	2	41,902	3
30	28,406	2	48,346	3
37	19,980	2	38,459	3
40	.....	..	36,698	3
60	30,599	2	27,040	3
Total No. of birds counted		30		81

SUMMARY

An ornithosis virus of low virulence was used to expose turkeys by the following routes: intratracheally, intraperitoneally, intranasally, and by contact. The body temperature rose 0.5 to 4.5 degrees following infection. An increase in leukocytes appeared to coincide with the temperature rise. Heavy mortality occurred in turkeys under 2 weeks of age, while older turkeys did not appear ill and no losses were found. The virus was isolated as soon as 72 hours following exposure and as late as 120 days after exposure. The complement-fixation test revealed a titer which reached its peak at 30 days and then began to decline.

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The Gross and Microscopic Pathology of an Ornithosis Virus of Low Virulence for Turkeys

C. GALE, D.V.M., Ph.D.; V. L. SANGER, D.V.M., M.S.;
B. S. POMEROY, D.V.M., Ph.D.

Wooster, Ohio

THE ORIGINAL isolation of an ornithosis virus from turkeys by Boney *et al.*[3] and subsequent isolations made by various workers,[2, 4, 7, 13, 14] established the turkey as a host and possible reservoir for the virus. The demonstration of avirulent strains of the ornithosis virus suggested the possibility of obtaining further information regarding pathogenicity, epizootiology, and other considerations. In previous reports,[8-10] the course of the disease, routes of infection, and other data have been reported. The gross and microscopic pathology are reported here.

MATERIALS AND METHODS

Turkeys were obtained from the station flock and held in isolation units from 1 day of age until used. The birds were exposed to the ornithosis virus by one of several routes: intranasal, intraperitoneal, intratracheal, and by direct contact. The birds when inoculated were 1 day to 6 months old with the majority of inoculations made at 6 weeks.

An ornithosis virus of low virulence for turkeys previously characterized in mice was used.[10] The inoculum used to infect the turkeys was either yolk sac material from infected chicken embryos or emulsified mouse tissue from infected mice. The spleen, liver, and ascitic fluid, collected from mice during the third consecutive virus passage, were macerated and diluted to a 10 per cent suspension in phosphate buffer. At different intervals following exposure to the ornithosis virus, birds were killed and examined for gross pathologic changes and tissues were obtained for microscopic examination. These tissues were fixed in neutral, buffered formalin-sodium chloride solution, embedded in paraffin, and sectioned 6 μ thick. Hematoxylin-eosin and Macchiavello's stains were used on all tissues.

RESULTS

Gross Pathology.—Intraperitoneal inoculation of turkeys resulted in the development of aerosacculitis, peritonitis, perihepatitis, and pericarditis. Birds under 2 weeks of age exhibited increased susceptibility with decreasing age; heavy mortalities occurred in this age group. Turkeys over 2 weeks of age showed no signs of sickness and no mortalities. In spite of a lack of clinical signs in older birds, marked pathologic lesions were found.

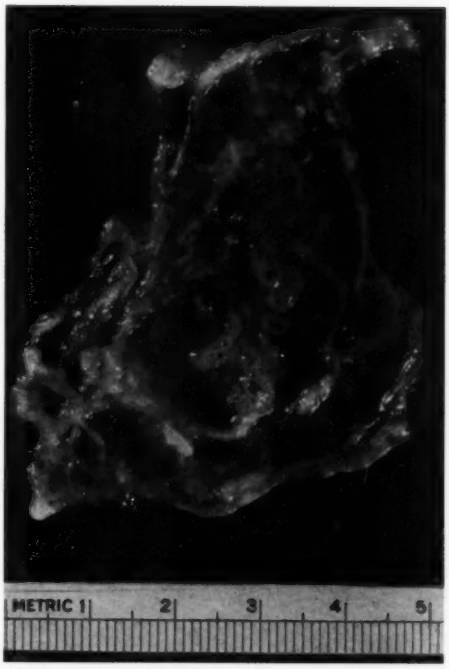

Fig. 1—Air sac from turkey killed nine days after inoculation with ornithosis virus, showing severe aerosacculitis. Notice the air sac is greatly thickened, cloudy, and edematous.

The first lesions were noticeable 72 hours after exposure when mild air sac infection and pericarditis were detected. The lesions were most extensive between the sixth and fourteenth days. Severe aerosacculitis, peri-

From the Ohio Agricultural Experiment Station, Wooster (Gale, Sanger), and the School of Veterinary Medicine, University of Minnesota, St. Paul (Pomeroy). Submitted to the Graduate School, University of Minnesota, as partial fulfillment for the requirements of the Ph.D. degree by the senior author, 1958.

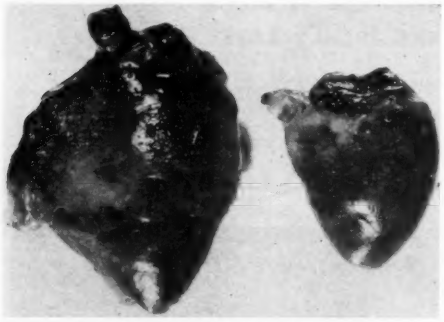

Fig. 2—Heart from turkey killed nine days after inoculation with ornithosis virus (left). The heart on the right is from a control turkey. Notice the cellular exudate beneath the reflected pericardial sac and enlargement of the heart of the inoculated turkey.

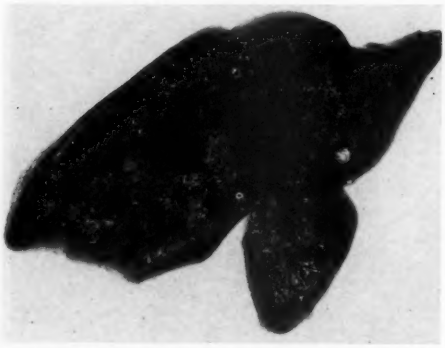

Fig. 3—Liver from turkey killed nine days after inoculation with ornithosis virus. Notice film of cellular exudate covering its surface.

tonitis, and pericarditis had developed in most cases by the ninth day (fig. 1). Cellular exudate was frequently observed in the pericardial sac (fig. 2) and, in other instances, the heart was flabby. Petechiae were found frequently during the acute stage of the disease and occasionally during the chronic state. The liver was frequently covered with a film of exudate (fig. 3). No other gross changes were found. No differences in size were noticed between the spleens of infected and control birds. After 14 days, the lesions began to regress; however, the air sac infection and peritonitis persisted in varying degrees.

Gross pathologic changes observed following intratracheal inoculations were similar to those of birds inoculated intraperitoneally except, at approximately the fifth day, the lungs exhibited areas of consolidation, congestion, and edema (fig. 4). There was severe air sac infection, pericarditis, perihepatitis, and peritonitis. Birds exposed by contact, when necropsied, were found to have lesions similar to birds inoculated intraperitoneally; however, the changes were not as extensive.

Intranasal inoculations resulted in mild aerosacculitis; no sinusitis was noticed.

Microscopic Pathology.—The microscopic pathologic changes of this experimental ornithosis were most prominent in the lungs, air sacs, and pericardium. The affected lung was consolidated as a result of cellular infiltration, edema, congestion, and hemorrhage. In the affected areas, parabronchi contained large masses of fibrin in

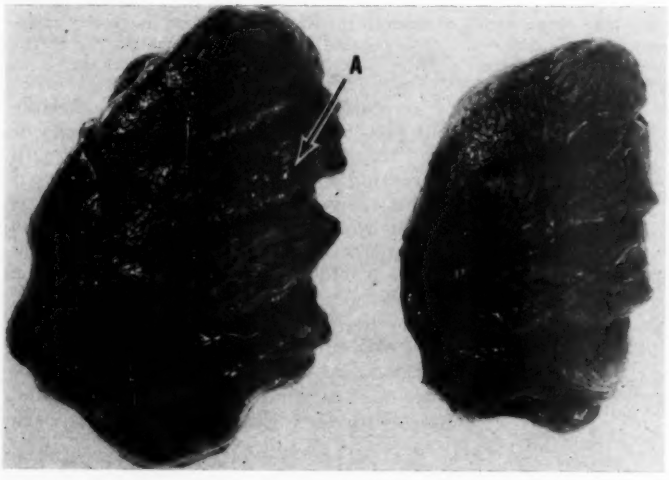

Fig. 4—The lung on left is from a turkey killed six days after intratracheal inoculation with ornithosis virus. Notice the grayish, consolidated, smooth edematous area involving almost half of the lung. Hemorrhage can be seen in the functional part at (A). The lung on right side is from a control turkey.

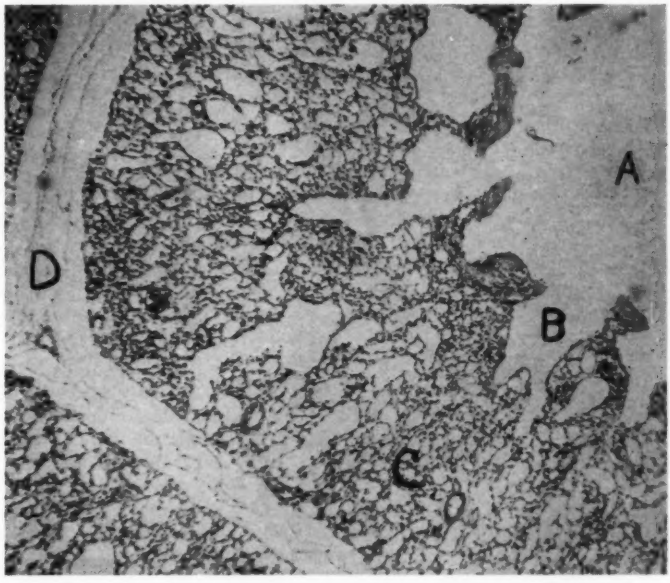

Fig. 5—Section of normal turkey lung: (A) parabronchus; (B) respiratory tubule; (C) air capillaries (alveoli); (D) interlobular septum. H & E stain; x 60.

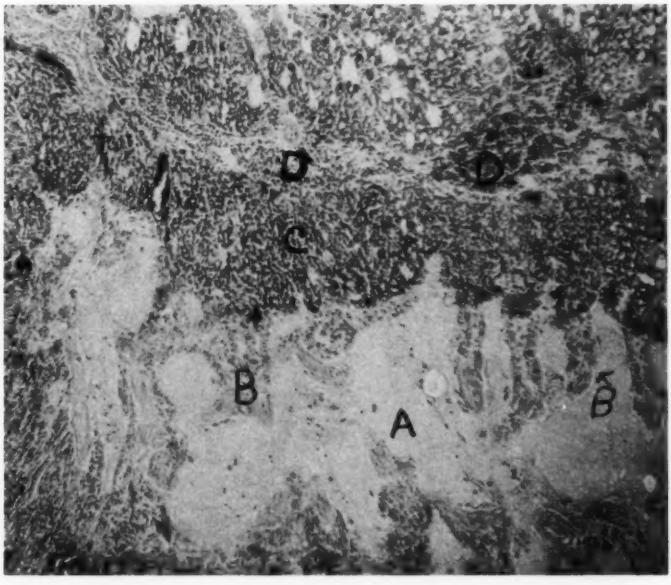

Fig. 6—Section of lung from a turkey killed six days after intratracheal inoculation with ornithosis virus. The parabronchus (A) is completely filled with edema fluid and a few cells. The walls of the parabronchus (B) are thickened and edematous. The air capillaries (alveoli) (C) are entirely filled with cellular exudate and are nonfunctional. The interlobular septum (D) is heavily infiltrated with heterophils and macrophages. H & E stain; x 60.

which were caught erythrocytes, heterophils, and phagocytes (fig. 5, 6). The bronchial epithelium was detached in some areas and cilia were lost from the parabronchial walls. The architecture of the lung was lost and alveolar capillaries were markedly congested; free hemorrhage was present and there were massive infiltrations of lymphocytes, phagocytes, and scattered plasma cells. Heterophils were numerous in these areas. Alveolar cells were swollen and pale. Necrosis was not present and fibrosis

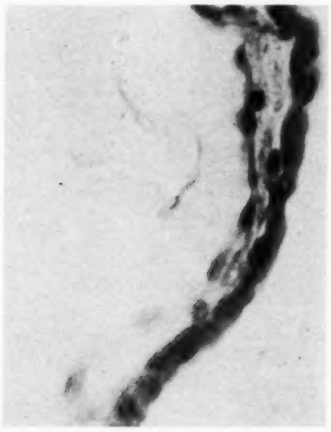

Fig. 7—Section of a normal air sac from control turkey. H & E stain; x 770.

was not apparent. In less severely affected areas of the lung, parabronchi and alveolar spaces remained open, but all capillaries were congested.

The air sac was greatly thickened and the free surface was covered with a cellular exudate composed of necrotic debris, heterophils, lymphocytes, and monocytes. The surface layer of epithelial cells was indistinct and many epithelial cells were absent. The character of the intermediate reticular layer was variable from one section of the membrane to another. In some areas, it was heavily infiltrated with lymphocytes, macrophages, and heterophils. Fibroblastic proliferation was marked and fibrinous clots were apparent throughout this layer. Small nests of epithelial cells had been trapped within the exudate beneath the free surface of the thickened membrane. Free hemor-

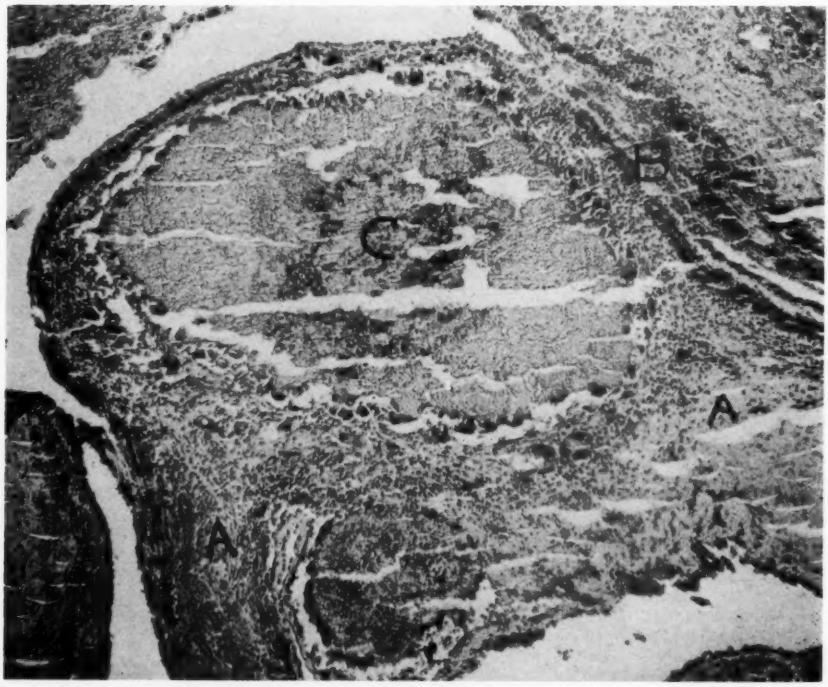

Fig. 8—Section of air sac from a turkey killed nine days after intratracheal inoculation with ornithosis virus. Notice the greatly thickened membrane (A) with cellular infiltration, fibrosis and necrosis. Adhesions have formed between two adjacent sections of the air sac (B). A very large abscess has formed within the air sac (C). H & E stain; x 80.

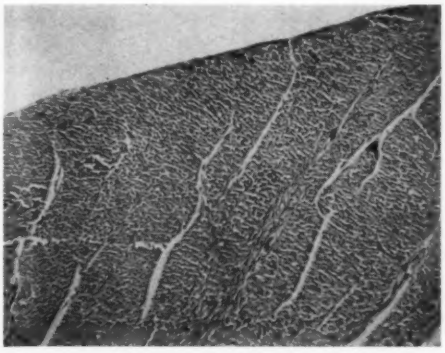

Fig. 9—Section of the wall of the heart from a control turkey. The epicardium forms thin dark line on surface of heart. H & E stain; x 110.

rhage had occurred in some places. In other areas of the membrane, the intermediate reticular layer had been completely replaced with hyalinized, necrotic debris. An occasional abscess containing caseous necrotic debris was present and was surrounded by a layer of giant cells. The air sac membrane was greatly thickened and folded so that adjoining areas were in contact and adhesions had formed at these points (fig. 8). Under these conditions, it would appear that the respiratory functions of the membrane would be completely lost (*see* normal air sac, fig. 7).

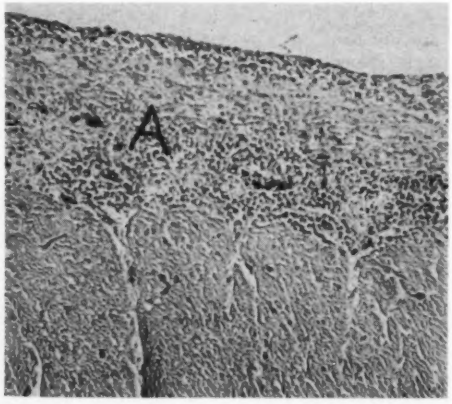

Fig. 10—Epicardium (A) of a turkey killed ten days after inoculation with the ornithosis virus. Notice the extreme thickening caused by leukocytic infiltration and fibroplasia. Some eosinophilic debris is present throughout the membrane. H & E stain; x 110.

The epicardium was greatly thickened, but edema was not present (fig. 9). The epicardium remained closely adherent to the myocardial surface, and it had been infilterated with large numbers of monocytes, macrophages, and a few lymphocytes. Heterophils were noticeably absent from these lesions. The free surface of the pericardium was covered with a layer of cells and granular eosinophilic debris (fig. 10). The myocardium was unaffected.

Chronic lesions in the heart from old adhesions exhibited mild focal epicarditis with considerable fibrosis and infiltration of monocytes resulting in thickening of the epicardium (fig. 11). Capillary hyperplasia was common (fig. 12).

Macchiavello's stained impression smears of the epicardium revealed the presence of many elementary bodies. These bodies were also found in the peritoneal exudates.

Discussion

Exposure to the virus resulted in subclinical infection in turkeys over 2 weeks of age. Although there was a lack of clinical signs, the infected turkeys exhibited distinct pathologic lesions resembling those found in infection with the more virulent strains of the virus. The most extensive lesions appeared in the lungs, air sacs, and pericardium. The gross appearance of the lesions was indistinguishable from those of chronic respiratory disease [22]; this observation has also been made regarding the virulent strains of ornithosis virus.[3]

Pericarditis was found in a large percentage of exposed turkeys, the degree of involvement being less than that reported with virulent strains.[1] Pericarditis similar in nature to that observed in ornithosis has also been noted in *Escherichia coli* infections in turkeys and chickens.[12] Absence of gross involvement of the liver and kidneys was noticed except for an exudate which covered the surface of these organs in some instances. Some liver involvement has been reported where more virulent viruses are concerned.[1, 6, 17] The degree of pathologic lesions might perhaps be attributed to the virulence of the strain of virus. No marked differences could be found between the spleens of infected and control birds; this observation would support other observations that splenomegaly does not hold the same relationship in ornithosis in turkeys as psittacosis does in free-flying

Fig. 11—Chronic focal epicarditis showing fibrosis and monocytic infiltration. Notice the swollen columnar epithelial cells on the surface. H & E stain; x 435.

birds.[6,17] Pneumonia was produced by this virus and was similar to that of the more virulent strains.[1,6,17] Previously, pneumonic lesions were not described as features of psittacosis in birds except for an occasional observation.[15,19,21]

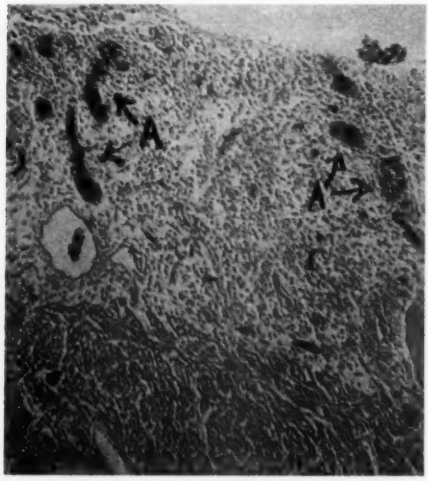

Fig. 12—Section of the epicardium of a turkey killed 30 days after inoculation with ornithosis virus. Notice the capillary hyperplasia (A) and fibrosis. The many dark specks throughout are fibroblasts and leukocytes. H & E stain; x 88.

An interesting fact about this disease is that no clinical signs of disease were apparent in turkeys over 2 weeks of age even though extensive pathologic lesions were present. In descriptions of natural outbreaks of the disease, turkeys were frequently observed to be ill, although not always.[18] Although the virus under investigation was of low virulence, this may be relative since high mortality occurred in birds under 2 weeks of age.

Human infection was reported to have occurred from exposure to this or similar strains of the ornithosis virus.[11] In that report, no severe epizootics of ornithosis had been observed in turkey flocks during three years of surveillance. The human infection was attributed to a mild and inapparent ornithosis in turkeys from a virus of low virulence.[11] Strains of ornithosis virus of low virulence have been previously reported.[16] However, human illness from exposure to these strains has not been reported.

The virus described here resembles the strains of low pathogenicity described by Page.[16] He found that isolates of low virulence were obtained from turkeys with a mild disease and low mortality, with no re-

ported human involvement. When tested in mice by the intravenous route, the isolates were found to be of low toxicity. The highly virulent viruses responsible for high mortality in turkeys and man were found to be highly toxic for mice. It has been postulated by Page [16] that the difference between high-virulent and low-virulent strains is associated with the ability of these viruses to produce toxins. The virus reported here was found to be of low toxicity for mice.

Davis *et al.*[5] compared nine strains of ornithosis and found considerable variation in pathogenicity. He suggested that psittacosis-ornithosis agents be classified according to pathogenicity rather than by hosts from which they were isolated. Strains of ornithosis virus of low virulence may be more widespread in the turkey population than is now recognized.

SUMMARY

The gross and microscopic pathologic lesions of turkeys exposed to an ornithosis virus of low virulence were reported. The pathologic lesions observed were pneumonia, pericarditis, perihepatitis, peritonitis, and aerosaculitis. Microscopic studies revealed marked cellular infiltration of the lung, with congestion and edema. The air sacs were greatly thickened, with marked cellular infiltration, fibrosis, and necrosis. The epicardium was thickened and infiltrated with monocytes, macrophages, and a few lymphocytes.

Chronic focal epicarditis and capillary hyperplasia were also found. Smears that were stained with Macchiavello's stain revealed the presence of elementary bodies. Birds under 2 weeks of age were found to be more susceptible; symptoms and losses increased in severity as the age decreased. Turkeys over 2 weeks of age showed no signs of illness.

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Recent Studies on the Virus of Infectious Bronchitis

C. H. CUNNINGHAM, D.V.M., Ph.D.

East Lansing, Michigan

EARLY STUDIES on infectious bronchitis were primarily concerned with disease manifestations and methods for the isolation and identification of the virus.[6] Recent research in multidisciplinary areas has served as the stimulus for more penetrating studies of the properties of the virus Within the last few years, findings of major importance to fundamental and applied virology have been reported. It is the purpose of the present contribution to review some of these findings and to consider possible areas for future investigation.

CELL (TISSUE) CULTURE

One of the most rewarding areas of investigation has been the use of cells derived from a variety of organs of the chicken embryo as well as mouse liver (Earle's L strain), monkey kidney,[9] and Hela cells [8] in tube, flask, and cover slip cultures in attempts to propagate infectious bronchitis virus (IBV). The medium most commonly used has been Hanks' balanced salt solution (BSS) containing normal serum of several species, chicken embryo extract, and lactalbumin hydrolysate, all in various combinations. Medium 597 containing BSS and nutrient additives has also been employed.[9]

The strains of IBV used almost exclusively have been the Connaught Laboratories vaccine R, Beaudette egg-adapted, Hofstad 33 and 97, Massachusetts, and Connecticut strains. Criteria of *in vitro* propagation of the virus in the intracellular and extracellular phases have been infectivity for chicken embryos, colorimetric changes of the medium, and cytopathic effects on stained and unstained cells.

Cells from the chicken embryo trachea and lung [1] and Hela cells [8] are considered to not support multiplication of the Beaudette [8] and Hofstad 33 and 97 strains of virus. The Connaught R and Beaudette strains do not infect mouse liver cells but do multiply in monkey kidney and chicken embryo liver and heart cells without the production of cytopathic effects.[9] The Massachusetts and Connecticut strains do not infect chicken embryo kidney cells.[3]

Despite reports to the contrary,[1,2] the Beaudette strain can be readily cultivated by serial passage in chicken embryo kidney,[3,7,9] liver, and heart,[9] whole embryo fibroblasts,[3,9] and isolated chorioallantoic membrane.[7,9,10,14] A few serial passages are necessary to produce virus of a high titer by adaptation from the original inoculum of virus-infected allantoic fluid. Production of virus ceases when the cells are destroyed. Thorough washing of chicken embryo liver or whole embryo fragments are necessary to remove maternal antibodies before the cells can be used for cultivation of the Beaudette strain. The Connaught R strain can be serially passaged in the chorioallantoic membrane but not in embryonic liver or whole embryo, even when the tissues are carefully washed to remove residual antibodies.[9]

The optimum conditions for propagation of the Beaudette strain in chorioallantoic membrane culture are incubation at 37 C. with the BSS at a pH of 7.0. The maximum yield of virus is obtained from cultures containing from 200 to 600 mg. of membrane per 20 ml. of BSS. Lesser yields are obtained from cultures containing 50 or 100 mg. of membrane. Cultures inoculated immediately after preparation, or incubated up to 105 hours after preparation, yield greater amounts of virus than cultures incubated 144 hours before inoculation.[14]

With chicken embryo kidney cultures, cytopathic effects are detectable within 24 hours.[3,7] By 72 hours there is complete destruction of the infected cells. Infection inhibits cellular metabolism as indicated by no lowering of the pH of the medium.

Microscopic examination of stained sections of the infected chorioallantoic membrane does not reveal specific cytologic

From the Department of Microbiology and Public Health, Michigan State University, East Lansing. Journal article No. 2496 of the Michigan Agricultural Experiment Station.

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changes that can be attributed to viral activity.[10]

Viral titration may be performed with chicken embryo kidney cultures by using cytopathic effects as revealed by microscopic examination[3] and by the plaque technique.[17] Plaques may be seen without the aid of the microscope before staining and are apparent in 16 to 18 hours. After three days, the plaques are 3 to 4 mm. in diameter. Comparison of titers obtained by the plaque count (PFP) and by embryo infectivity (ELP) shows a PFP/ELP ratio of 4 by assuming from the Poisson distribution that one embryo $l.d._{50}$ equals 0.7 egg particles.[17]

Growth curves for the Beaudette and Connaught R strains in chorioallantoic membrane culture, using diluted inoculum and testing for infectivity in chicken embryos, show that for both viruses the lag phase or absorption period is four to eight hours, with complete disappearance of the virus from the extracellular fluid. The latent period for the Beaudette virus varies from 12 to 18 hours and for the Connaught R virus from 20 to 26 hours. The log phase maximum is at 48 hours for the Beaudette strain and 40 hours for the Connaught R strain, followed by a decline phase. New virus is formed for at least eight days with the Beaudette strain and for three days with the Connaught R strain.[9]

Using the Beaudette virus undiluted, the lag phase is eight hours without complete disappearance of the virus from the extracellular fluid. The maximum of the log phase is reached after 60 hours, followed by a primary decline phase, a stationary phase, and a secondary decline phase. New virus is formed for at least ten days.[14] While growth occurs in Hanks' BSS from which potassium, calcium, magnesium, or glucose have been omitted, viral concentration is lower and the maximum of the log phase is at 36 hours, as contrasted to 60 hours with the complete BSS.[10]

The Beaudette virus, cultivated in chicken embryo kidney cells and titrated according to the cytopathic effect produced in subcultures in kidney cells, shows disappearance of the virus from the extracellular fluid within four hours. The virus reappears within 16 hours, and the maximum yield is reached at 48 hours and maintained until 72 hours; it then declines. Virus is produced for at least five days. The same virus tested in chicken embryos follows a similar pattern but at approximately 10^3 higher infectivity.[3]

HEMAGGLUTINATION

Infectious bronchitis virus modified by trypsin and the subsequent addition of egg white or soybean trypsin inhibitor contains an active hemagglutinative agent for chicken erythrocytes but not for those of several other species of animals. Maximum hemagglutination is obtained with virus in low egg passage and varies with viral concentration. Hemagglutination titers are generally 2,560 or 5,120 with representative strains such as Hofstad 33, 104, and 314 and Massachusetts viruses. With the Beaudette strain, the reaction is minimal or negative. The rate and degree of adsorption of the hemagglutinin to chicken erythrocytes varies directly with temperature. Elution, which does not occur spontaneously, varies indirectly with temperature. Modification of IBV by trypsin indicates that arginine or lysine, or both, may be present in the viral protein. Trypsinized IBV probably represents a true enzyme-substrate complex, and the resultant modification of the virus provides a product which is adsorbed to the cell surface and causes hemagglutination. Trypsin-modified cells do not produce hemagglutination with untreated IBV. No enzymic activity of the virus itself is involved in hemagglutinative activity, since heat-inactivated virus participates in the test. With IBV hemagglutinin, cells are reagglutinable. Specificity of the hemagglutinin has not been demonstrated by inhibition with anti-IBV serum. Hemagglutination is readily inhibited by negative and anti-IBV serum, ovomucoid, and normal and viral-infected allantoic fluid.[5]

THERMOSTABILITY

Strains of IBV vary in their thermostability at 56 C.[11] Virus in low egg passage may be inactivated over a range of 30 to 150 minutes, as tested by infectivity for chicken embryos.[16] There is some question as to the correlation between the concentration of the virus and thermostability. Virus in high egg passage, such as the Beaudette strain, is inactivated within a few minutes.

Specific reaction rates of inactivation of undiluted virus-infected allantoic fluid of low egg passage, as calculated from $\log_{10}$

residual viral infectivity, show that the three-halves order reaction, in which the reciprocal of the square root of the concentration $\left(\frac{1}{\sqrt{C}}\right)$ is proportional to time, best interprets the reaction and is statistically significant. This reaction is complex and indicates that IBV is inhomogeneous with respect to centers of thermal sensitivity and contains more than one type of viral particle, each of which varies in thermostability.

Since a three-halves order reaction is indicative of a bimolecular reaction, it may be assumed that IBV exists in two phases: the original (O) or non-egg-adapted phase, in which the particles retain their original identity and are thermostabile; and the derivative (D) or egg-adapted phase, in which the particles have been derived as a result of embryo culture and are thermolabile.

The kinetic process of thermal inactivation of a mixed population of O and D phases may be divided into two first-order specific reaction rate constants with D phase having the higher rate and greater thermolability. The thermostability of the mixed population varies directly with the O/D ratio. By extrapolation of the D phase constant to the base line and heating the mixture for the indicated time, it is possible to inactivate the D phase and selectively retain the O phase. The reaction rate constant of the O phase as a first-order reaction is in close agreement with the constant for the O phase of the mixed O-D population. By means of the limiting dilution technique, it is possible to maintain the O phase characteristics alone through at least five serial passages in eggs.[16]

When the Beaudette virus is suspended in BSS alone at 37 C., inactivation occurs within nine[14] to 16 hours.[9] Similarly, the Connaught virus is inactivated within 30 hours.[9]

The specific reaction rate constant for thermal inactivation of the Beaudette strain in chorioallantoic membrane culture and tested for infectivity in chicken embryos is $10^{0.1}$/week^{-1} at 25 C. and proceeds according to a first-order reaction. At 45 C., the constant is $10^{0.15}$/minute^{-1} for the first 30 minutes and $10^{0.005}$/minute^{-1} for the next 90 minutes. The activation energy for inactivation of the Beaudette strain is 21.29×10^3 calories/mol.

IMMUNOGENIC TYPES

Recognition of antigenic differences[12] and immunologic types,[13] which may well be considered as serotypes, affords further characterization of IBV. The stimulus for investigations of serotypes was based on the finding that the Beaudette strain, which is commonly used as the antigen for neutralization tests in chicken embryos, did not reveal antibodies in chickens which had recovered from infection with certain isolates of IBV. The Beaudette strain is nonimmunogenic, and effective anti-serum cannot be produced. As determined by reciprocal neutralization tests in chicken embryos and in chickens, cross immunization following challenge, and histologic reactions of the trachea, there is agreement that at least two antigenic and immunogenic types of IBV, designated as Connecticut and Massachusetts, exist.[13] There is the possibility that additional types may be defined.[12] Considerable variation may be detected in the neutralization indexes of antigenically related isolates.

Of particular interest in analysis of serotypes is the neutralization of the Beaudette strain in chicken embryo kidney cell cultures, using undiluted antiserums against the Connecticut and Massachusetts strains. The neutralizing capacity of these serums is lost when they are diluted 1:10. In parallel neutralization tests in embryos, only the homologous serums are capable of neutralization. Antibodies against the Connecticut strain are only rarely detected by the Beaudette virus.[3]

HISTOLOGY

The Connecticut and Massachusetts viruses produce similar histopathologic lesions in the trachea following inoculation by this route. The tracheal mucosa undergoes cyclic changes in three main phases: (1) acute phase (1 to 3 days)—epithelial hypertrophy and marked edema; (2) reparative phase (6 to 9 days)—epithelial hyperplasia and marked cellularity of the propria; and (3) immune phase (12 to 18 days)—restoration of the epithelium and either follicular or mild, focal, diffuse lymphoid infiltration of the propria.

Histopathologic examinations of the trachea are considered to be superior to clinical examination as a means of assessing "takes." The advantages are objective

examination, permanent record, availability of results within two days postinoculation, and need for few birds to be held in isolation. The method is applicable to immunity tests for IBV in field specimens. When chickens inoculated with serum-virus mixtures are subjected to histologic examination two days postinoculation, those inoculated with homologous mixtures do not present lesions, whereas those given heterologous mixtures do present lesions.

Miscellaneous Properties

Additional studies have yielded much information on some basic properties of the Hofstad 33 and 97 and Beaudette strains.[1] Normal or infected allantoic fluids from 13-day chicken embryos appear by agar diffusion analysis to be antigenically similar. There are a minimum of seven precipitating antigens of a protein nature in viral fluid, of which none can be ascribed to the virus, and five antigens in normal fluid. The antigens are heat-labile and precipitated by trichloracetic acid. The number of precipitating materials is not reduced by extraction with ether or by oxidation with potassium periodate. Ficin reduces the density of the zones without a reduction in the number and trypsin reduces the number of zones.

The virus is a sphere of 120 mμ and is only partially sedimented by ultracentrifugation. Amorphous material observed by electron microscopy may be removed by treatment with trypsin. The density reading is between 1.16 and 1.15 in sucrose solutions and between 1.14 and 1.13 in glycerine solutions.

Trypsin does not inactivate the infectious portion of the virus, or release active virus from serum-virus mixtures, but it does inactivate antibodies in the absence of virus. Ammonium phosphate gel in phosphate buffer at various pH and molar levels does not adsorb the virus.

Ammonium sulfate in a 30, 50, or 70 per cent saturated solution precipitates the virus. Fifty per cent of the virus is removed with the thermodependent precipitate of allantoic fluid at 4 C. or below.

Viral infectivity is retained following lyophilization at 8 to 10 μ mercury pressure for 12 hours and sealed *in vacuo*. The following suspended mediums maintain the titer for at least ten days: 10 per cent glucose, 20 per cent inactivated horse serum, tryptose phosphate broth, and 10 per cent skim milk. A tenfold decrease occurs with 20 per cent inactivated bovine serum, 1.5 per cent bovine albumin, 1.0 per cent lactalbumin, 10 per cent sucrose, or undiluted allantoic fluid. A hundredfold decrease occurs with 0.01 M phosphate buffer, 10 per cent fructose, or 3 per cent gum acacia.

Treatment with *beta*-propiolactone in a final concentration of 0.025 per cent at 37 C. for two hours renders IBV noninfectious for chicken embryos, but it is immunogenic for chickens as shown by antibody response of recipients and their resistance to challenge.[4]

The following physical-chemical treatments do not inactivate IBV as tested for infectivity in chicken embryos: HCl, 1.0 per cent, pH 2.0, 60 minutes at room temperature; cresol, 1.0 per cent, 60 minutes at 25 C.; ethyl ether, 20.0 per cent, 24 hours at 5 C.; phenol, 1.0 per cent, 60 minutes at 25 C.; NaOH, 1.0 per cent, pH 12.0, 60 minutes at room temperature; thimerosal, 0.2 per cent, 60 minutes at 25 C.; erythromycin, 0.5 mg., 10 minutes at 25 C.; and phenyl mercuric borate, 0.01 per cent, 15 minutes at 25 C. At 56 C., the virus is inactivated within five minutes.[15]

Chemical fractionation for isolation of ribonucleic acid has failed to reveal the infectivity portion of the virus.[14]

Discussion

Research leading to the present knowledge of infectious bronchitis and of the causative agent has traveled the familiar pathways of investigations on infectious diseases. So frequently, the properties of the etiologic agent are the last to receive attention, and the information gained is used to interpret previous success or failure in the areas of clinical studies, isolation and identification of the agent, and immunization. The recent emergence of information on the properties of IBV has reached the point where progress will be dictated as much or more by fundamental research as by the demands of the applied areas, and will be more than adequate to handle practical problems.

Successful propagation of IBV in cell culture provides a new means for exploration of the virus-cell relationship through utilization of the newer techniques of the physical and biological sciences. One of the problems is understanding the metabolism of

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the host and the intracellular mechanism of the virus as an infectious agent and as an antigen.

The differences in neutralization of the Beaudette strain by Connecticut- and Massachusetts-type antiserums in cell and embryo cultures emphasize the need for further studies on the antigenic and immunogenic properties of IBV strains and the specificity of antigen-antibody reactions. The significance of the virus-cell interaction and of the sensitivity of detection of infection by these culture systems should be investigated.

Separation of the O phase of IBV from a mixed O-D population may offer a possible means for genetic analysis of properties, such as antigenicity and immunogenicity, that are altered as a result of serial passage of the virus in eggs. The diphasic type of inactivation of the Beaudette strain cultivated in isolated chorioallantoic membrane may be a reflection of a minority population of retained O-phase virus which is more readily detected in a synthetic medium as compared to allantoic fluid, which contains protective colloids such as proteins and mucopolysaccharides.

The intracellular supplies of the ions and glucose contained in Hanks' BSS are sufficient to support viral synthesis in the isolated chorioallantoic membrane at a reduced rate and for a shorter time when they are omitted from the extracellular fluid. This may be a reflection of inhibition of viral production through a lack of enzymic activation by the ions and reduced energy through the metabolic pathways of glycolysis and the Krebs cycle.

While there is definite evidence that trypsin-modified IBV contains a hemagglutinative agent, further studies are necessary to establish the specificity of the reaction by inhibition with anti-IBV serum.

Agar-diffusion analysis of IBV and antiserums deserves further investigation.

SUMMARY

1) The Beaudette strain of infectious bronchitis virus (IBV) can be readily cultivated by serial passage in cultures of chicken embryo kidney cells and in the isolated chorioallantoic membrane. Specific cytologic changes that can be attributed to viral activity are not observed in the chorioallantoic membrane. Viral titration may be performed with chicken embryo kidney cultures by using cytopathic effects as revealed in microscopic examination and by the plaque technique.

2) The omission of ions and glucose from Hanks' balanced salt solution deleteriously affects, but does not completely inhibit, viral synthesis in the isolated chorioallantoic membrane.

3) There are at least two antigenic and immunogenic types of IBV, designated as Connecticut and Massachusetts types, and there is the possibility that additional types may be defined.

4) Trypsin-modified IBV in low egg passage contains an hemagglutinative agent.

5) Histologic reactions of the trachea may be used to assay infection and neutralization by antibody.

6) Embryo-propagated IBV can be separated into original phase and derivative phase particles by differential thermostability. By means of the limiting dilution technique, it is possible to maintain the original phase characteristics through serial passage in eggs.

7) By means of agar-diffusion analysis, antigen-antibody complexes of IBV and antiserum may be detected. None of the precipitating antigens can be specifically ascribed to IBV.

8) Other miscellaneous properties of IBV are reported.

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Comparative Study of Three Isolates with the Original Feline Viral Rhinotracheitis Virus

Major R. A. CRANDELL, V.C., U.S.A.F.; Captain J. R. GANAWAY, V.C., U.S.A.F.;
Lieutenant W. H. NIEMANN, V.C., U.S. Army;
Colonel F. D. MAURER, V.C., U.S. Army

Washington, D.C.

In 1958, a new feline virus, associated with intranuclear inclusion bodies, which is responsible for an upper respiratory infection of the domestic cat was reported.[2] In a subsequent report,[1] the name "feline viral rhinotracheitis" (FVR) was proposed for this disease. Since the initial report, several additional viruses have been isolated from the domestic cat, some of which came from cats clinically ill.

Studies were initiated to identify and group the different isolates in respect to their relationship to each other. For convenience, they have been placed into two groups: (1) those associated with inclusion bodies, and (2) those in which inclusion or elementary bodies have not been demonstrated.

This paper records results with three new isolates in group 1, since they are associated with inclusion bodies. These three viruses were isolated from cats exhibiting clinical signs similar to those of FVR. A comparison of the three isolates is made in terms of their relationship to the original FVR isolate.

The objective of the study was to determine whether any differences could be found between the isolates in respect to their cytopathogenicity and plaque-forming abilities in cultures of feline renal cells and by serum-neutralization tests.

Materials and Methods

Tissue Cultures.—Primary cultures of feline renal cells were prepared on 11- by 22-mm. coverslips in Leighton tubes, 15- by 155-mm. culture tubes, and 4-oz. prescription bottles.[4] The cells were grown in a nutrient medium consisting of 0.5 per cent lactalbumin hydrolysate in modified Hanks' salt solution containing 0.01 M.Tris (hydroxymethyl aminomethane), adjusted to pH 7.4, and 10.0 per cent lamb serum. The maintenance fluid used at the time of inoculation was lactalbumin hydrolysate with the addition of 3 per cent lamb serum. Streptomycin (0.5 mg./ml.), penicillin (500 units/ml.), and mycostatin (100 units/ml.) were incorporated in all mediums.

TABLE 1—Results of Cross-Neutralization Tests in Tissue Culture of the Four Isolates Utilizing 50 to 500 $t.c.i.d._{50}$ Doses*

Virus	Antiserum			
	FVR-1	FVR-2	FVR-3	P-001**
FVR-1	64	32	32	64
FVR-2	64	32	32	64
FVR-3	128†	32	32	128†
P-001**	64	32	32	64

* Serum titers are expressed as the reciprocal of the highest serum dilution which inhibited the cytopathogenic change in two or more tubes. ** Pullman 001. † The challenge dose in these two serum-neutralization tests was calculated to be 20 $t.c.i.d._{50}$.

Viruses.—The viruses were isolated in the manner previously described[2] and designated as FVR-1, FVR-2, FVR-3, and Pullman 001. The original isolate, FVR-1, was obtained from a cat procured from Pennsylvania. Viruses FVR-2 and FVR-3 were obtained from cats raised in Maryland but maintained in separate colonies. The Pullman 001 agent was isolated from the Fur Animal Disease Station cat colony at Pullman, Wash.* At the time of study, all viruses had been passed a comparable number of times in tissue culture.

Virus Titration.—Serial tenfold dilutions of tissue-culture fluid containing the virus were prepared, using nutrient fluid as the diluent; 0.1 ml. of each dilution was inoculated into each of four feline kidney tissue-culture tubes. The inoculated tubes were observed for six days for cytopathogenic change (CPC). The end point was calculated by the Reed and Muench method[5] and expressed as the 50 per cent tissue-culture infective dose ($t.c.i.d._{50}$) per 0.1 ml. of tissue-culture fluid.

Technique for Plaque Formation.—Plaque studies were performed using the prescription bottle method.[3] Just before use, the nutrient fluid was removed, 0.3 ml. of the virus was added in dilutions of 10^{-4} and 10^{-5}, and the cultures were incubated at 37 C. for one hour. After virus fixation, the cultures were overlaid with 10 ml. of melted agar (1.5%) in Earl's salt solution containing 2 per cent lamb serum and 0.0017 per cent neutral red. After the agar solidified, the bottles were incu-

From the Veterinary Pathology Division, Armed Forces Institute of Pathology, Washington 25, D.C.

* Supplied through the courtesy of Dr. John R. Gorham, Pullman, Wash.

bated at 37 C., with the cell layer uppermost. Plaque development was observed daily by examining the bottles under a dissecting microscope.

Serum-Neutralization Tests.—Serial twofold dilutions of serum inactivated by heat at 56 C. for 30 minutes were prepared, using nutrient fluid as diluent. An equal amount of virus suspension, diluted to contain between 100 and 1,000 tissue-culture infective doses, was added to each dilution. The serum-virus mixtures were then incubated at room temperature for one hour. Following incubation, 0.1 ml. of each serum-virus mixture was inoculated into each of three feline kidney-culture tubes. The tubes were incubated at 37 C., and daily observations were made for CPC. The presence of neutralizing antibodies was indicated by the absence of CPC. Serum-neutralization tests for the detection of nonspecific inhibition of CPC were conducted, using preinoculation rabbit serum and rabbit serum prepared against noninfected tissue-culture fluid. Cross-neutralization tests were performed with all viruses and their antiserums.

Preparations for Studying Cytopathology.—Feline renal cell cultures in Leighton tubes with coverslips were inoculated with 0.1 ml. of the respective viruses. Following inoculation, all cultures were incubated at 37 C. and observed with a light microscope at a magnification of 100 for presence of CPC. The coverslips were fixed in Bouin's fluid and stained with hematoxylin and eosin.

Immune Serum Production.—Immune serum was prepared against the four viruses in young adult New Zealand rabbits.

Each animal received three intravenous inoculations of 5 ml. of undiluted, infected tissue-culture fluid at weekly intervals. Blood samples were taken prior to inoculation and again one week following the last injection. As a control for nonspecific inhibition of CPC, 1 animal received tissue-culture fluid from uninfected cultures.

RESULTS

The constant features of the cytopathogenicity of all four isolates studied were the occurrence in stained preparations of intranuclear inclusion bodies (fig. 1) and the formation of syncytia or multinucleated giant cells (fig. 2). With each virus, the CPC was the same. With a comparable t.c.i.d.$_{50}$ dose of each virus, the time of occurrence and development of the lesions were similar. Replicate titrations of the four viruses in cultures of feline renal cells showed a t.c.i.d.$_{50}$ between $10^{-5.5}$ and 10^{-6}. In each instance, there was a slight reduction in the metabolic activity in the infected cultures as measured by the pH of the nutrient fluid.

Macroscopic plaques measuring 1 to 3 mm. were evident in feline renal cultures overlaid with agar on the sixth day following inoculation. The size of the plaque continued to increase and reached a maximum diameter of 1 cm. by the twelfth day. They appeared as well-circumscribed areas with a slightly irregular border. The plaque formation of each of the four viruses was uniform in size and morphology (fig. 3). However, with a greater dose of virus, overlapping occurred with adjacent plaques.

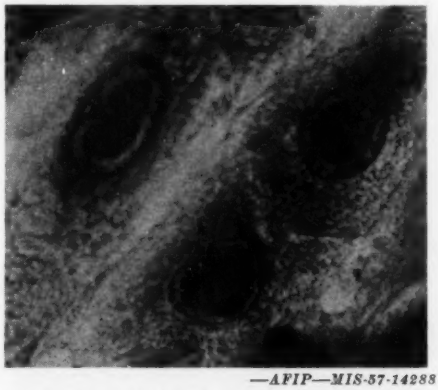

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Fig. 1—Characteristic intranuclear inclusion bodies of one of the four isolates (FVR-1) in culture of feline renal cells. H & E stain; x 1,100.

Results of the serum-neutralization tests are presented (table 1). The serum titers are expressed as the final serum dilution. The end point was determined by the serum dilution which completely inhibited the CPC in two or more tubes. Comparable titers of

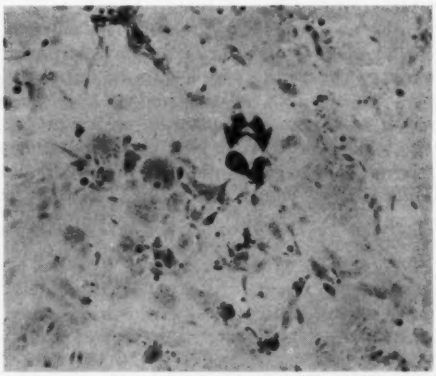

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Fig. 2—Characteristic cytopathogenic changes in cultures of feline renal cells infected with the Pullman 001 isolate. Note giant cells. H & E stain; x 60.

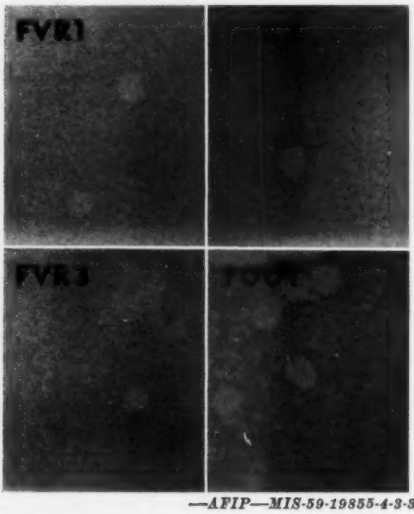

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Fig. 3—Plaque morphology of the four feline isolates in cultures of feline renal cells; FVR-1, 2, and 3 were photographed on the eighth day and Pullman 001 on the ninth day following inoculation.

neutralizing capacity were obtained from the serum prepared against each virus. Each serum possessed an equal degree of protection against its homologous virus and also when tested against the other three viruses. Both the preinoculation serum and the serum prepared against uninfected tisue-culture fluid failed to inhibit the CPC.

DISCUSSION

No significant differences between these viruses were demonstrated by the methods employed in this study. Their cytopathogenicity for feline renal cells in culture is indistinguishable. Intranuclear inclusion bodies and giant cells are a consistent feature of their cytopathogenicity. The viruses produce macroscopic plaques in cultures of feline cells overlaid with agar. The plaques are similar in regard to their time of appearance, size, and shape.

There was no evidence to indicate that the virus isolates were different antigenically. Each serum neutralized the other viruses to the same degree, as it did the homologous virus. When the challenge dose was of lower magnitude, the serum titer was correspondingly higher with each serum. Sharp neutralizing end points were obtained, and breaks did not occur. For this reason, it is believed that the antigen-antibody complex is not only lasting but also highly specific in nature.

The four viruses were isolated from naturally occurring cases of clinically identical respiratory diseases in cats during the acute phase of illness.

In addition to these natural cases, the disease has been reproduced under experimental conditions.[2] Clinical disease was produced and intranuclear inclusion bodies demonstrated in necropsy material of the upper respiratory tract.

The natural cases reported here represent different geographic regions of the United States. The fact that the virus was isolated on both the East and West Coasts suggests this pathogenic agent is widely distributed throughout this country.

SUMMARY

Three separate virus isolates, each obtained from clinically ill domestic cats, were compared with the original virus of feline viral rhinotracheitis (FVR) in regard to their cytopathogenicity and ability to produce plaques in culture of feline renal cells and their serologic relationship.

There were no distinguishing characteristics in their behavior in the systems investigated to indicate that the four isolates were different.

It is suggested that FVR may be widespread in the United States. Further, since this virus is the cause of respiratory disease, it has the potential of being a major problem in feline practice, particularly in cat colonies.

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Resistance of the Opossum to Rabies Virus

P. D. BEAMER, D.V.M., Ph.D.; C. O. MOHR, Ph.D.;
T. R. B. BARR, M.R.C.V.S., M.V.Sc.

Urbana, Illinois

THE COMMON American opossum *(Didelphis marsupialis virginiana)* is reported occasionally as being infected with rabies. The Committee on Animal Health of the National Research Council [4] reported 1 case among hundreds of wild animals examined in the United States in 1944. Gier [5] stated that opossums have been suspected of having rabies, but that the disease apparently does not become epizootic among them and, hence, does not seriously affect their populations.

In recent years, more cases of rabies in wildlife, including opossums, have been reported than previously,[9] probably because of increasing interest on the part of the public and of scientific investigators. However, in many areas where trappers are accustomed to catching at least as many opossums as skunks and four or five times as many opossums as foxes, only 1 or 2 rabid opossums are reported annually.[9] The number of opossums submitted for test remains low and rabies outbreaks appear to be confined to Canidae, skunks, and occasionally raccoons.

This does not, however, rule out the opossum as a possible natural host of rabies. Indeed, certain of its characteristics and the occurrence of some rabies infection in the opossum qualify it as a possible natural reservoir. Opossums are numerous and rabies in them appears to be atypical.

Other viral and rickettsial diseases seem also to take an atypical course in opossums. Travassos [8] reported that opossums *(D. marsupialis aurita)* develop inapparent infections of American spotted fever after inoculation and that viable rickettsiae persists in them for long periods. Brigham [1] showed that the opossum is susceptible to murine typhus but that the organism apparently caused no elevation of temperature. Bugher [2] reported that opossums exhibit a low order of susceptibility to yellow fever, that circulating virus levels are low, and that antibody production is weak.

These facts and the obvious need to study the possible host status of so numerous an animal prompted us to test the susceptibility of the opossum to rabies virus under controlled conditions.

From the College of Veterinary Medicine, University of Illinois, and the Illinois Natural History Survey, Urbana.

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MATERIALS AND METHODS

Thirty-four wild, trapped opossums were used in these observations. They were trapped in various parts of Illinois as a part of other studies conducted by the Illinois State Natural History Survey. This method of procurement resulted in wide variation in age, state of nutrition, and general health. These variations may have exerted some influence on the results of the experiments.

Opossums were held in individual cages and were provided free access to water and a commercial dry dog food.

Albino test mice were purchased * at 18 to 21 days of age; a few were raised in our animal colony.

Three strains of rabies virus were used. The first strain was isolated from a skunk *(Mephitis mephitis)* submitted to the Illinois State Diagnostic and Research Laboratory, Urbana. This strain produced well-defined Negri bodies in the naturally infected skunk and in mice exposed by intracerebral inoculation. The usual incubation period in mice was eight to ten days. The mouse $l.d._{50}$ of infective mouse brain was calculated as $10^{-4.6}$ for first mouse passage, and $10^{-6.2}$ for a pool of the second and third mouse passages according to the method of Habel.[6] The second strain was secured from Dr. Marlin Kleckner, Newton Field Station, Newton, Ga., who had isolated it from a fox *(Urocyon cinereoargenteus)*. The mouse $l.d._{50}$ was calculated as $10^{-4.5}$. The third strain was the standard challenge virus (CVS) strain of fixed virus secured from Dr. Karl Habel, National Institutes of Health, Bethesda, Md. The mouse $l.d._{50}$ was calculated as $10^{-6.9}$.

Opossums were restrained and inoculated as indicated in each experiment. When intracerebral inoculations were made, the skull was first trephined with a small metal drill. All mice were inoculated intracerebrally under light ether anesthesia.

The hippocampi from opossums were prepared for microscopic examination by paraffin section and a rapid smear technique. Sections were fixed in 10 per cent neutral formalin and Zenker's fluid, paraffin-impregnated in an autotechnicon and sectioned at 5 μ. All sections were stained with hematoxylin and eosin.[7] Smears were prepared from the hippocampi of opossums by crushing a bit of tissue, 3 to 5 mm. in diameter, on a 1 by 3-inch glass slide with a No. 2 glass coverslip; smearing it to produce a film approximately ¾ x 2 inches; and fixing it for three minutes in absolute methyl

* From the National Laboratory Animal Co., Creve Coeur, Mo.

alcohol. The smears were stained by a modification of Williams method, Coffin.[3]

Negri bodies were well demonstrated by this method. They stained a deep magenta. The inner-bodies were clearly visible when examined with the oil immersion objective.

EXPERIMENTAL

Experiment 1.—Sixteen opossums were used in this experiment. The procedure of inoculation is illustrated (fig. 1).

Six opossums were inoculated deeply into the masseter muscle with 40,000 mouse l.d.$_{50}$ units of skunk origin virus in the form of 0.25 ml. of a 20 per cent suspension of a pool of mouse brain and skunk brain. Saliva swabs were taken at ten-day intervals beginning 20 or 30 days after inoculation and continuing until death of the animal. These were tested for the presence of rabies virus by mouse intracerebral inoculation. All were negative. Opossum 2 died on the 69th postinoculation (PI) day. Mouse inoculation tests and histologic examination of the brain were negative for rabies. The remaining 5 opossums were killed on PI day 134. Mouse inoculation tests and histopathologic examinations were negative for rabies.

One milliliter of 20 per cent brain suspension from opossum 2 was inoculated deeply into the masseter muscle of opossum 11. Opossum 11 died 23 days later. Mouse-inoculation tests and histopathologic examination of the brain were negative for rabies.

Opossums 2 and 11 exhibited signs of central nervous disturbance before death. These signs included paralysis, gripping of the wire of their cages with their teeth, and refusal of food and water.

Brain tissue from opossum 11 was inoculated intracerebrally into opossum 12. Opossum 12 was killed 97 days later. Attempts to recover rabies virus by intracerebral mouse inoculation were negative.

Opossums 8, 9, and 10 were inoculated by a similar procedure using the same strain of virus after two and three mouse passages. These opossums were given 634,000 mouse l.d.$_{50}$ units of rabies virus into the masseter muscle. Opossums 8 and 10 were killed on PI day 126. Mouse inoculation tests and histopathologic examinations were nega-

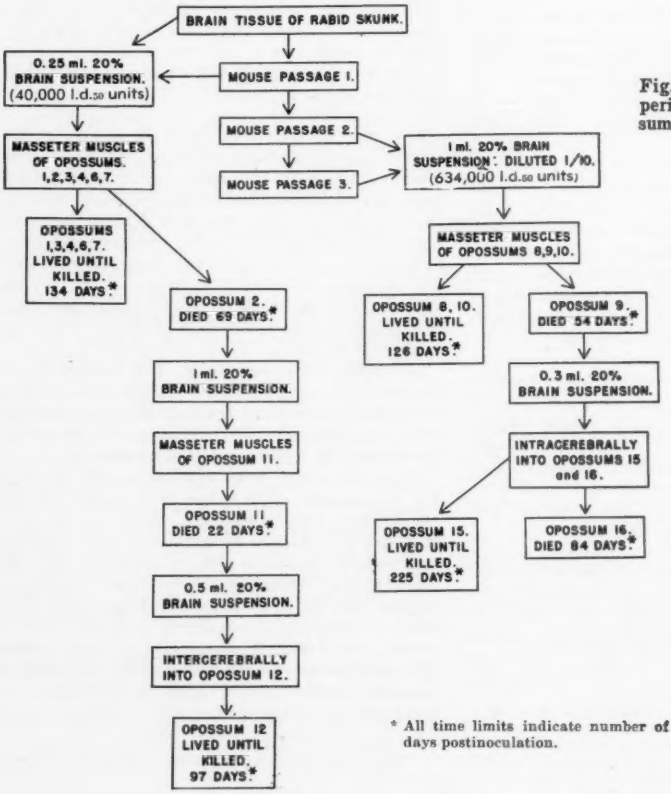
BRAIN TISSUE OF RABID SKUNK.
0.25 ml. 20% BRAIN SUSPENSION. (40,000 l.d.50 units)
MOUSE PASSAGE 1.
MOUSE PASSAGE 2.
MOUSE PASSAGE 3.
1 ml. 20% BRAIN SUSPENSION: DILUTED 1/10. (634,000 l.d.50 units)
MASSETER MUSCLES OF OPOSSUMS. 1,2,3,4,6,7.
OPOSSUMS 1,3,4,6,7. LIVED UNTIL KILLED. 134 DAYS.*
OPOSSUM 2. DIED 69 DAYS.*
1 ml. 20% BRAIN SUSPENSION.
MASSETER MUSCLES OF OPOSSUM 11.
OPOSSUM 11. DIED 22 DAYS.*
0.5 ml. 20% BRAIN SUSPENSION.
INTERCEREBRALLY INTO OPOSSUM 12.
OPOSSUM 12 LIVED UNTIL KILLED. 97 DAYS.*
MASSETER MUSCLES OF OPOSSUMS 8,9,10.
OPOSSUM 8, 10. LIVED UNTIL KILLED. 126 DAYS.*
OPOSSUM 9. DIED 54 DAYS.*
0.3 ml. 20% BRAIN SUSPENSION.
INTRACEREBRALLY INTO OPOSSUMS 15 and 16.
OPOSSUM 15. LIVED UNTIL KILLED. 225 DAYS.*
OPOSSUM 16. DIED 84 DAYS.*

Fig. 1—Procedure used in experiment I to inoculate opossums with rabies virus isolated from a skunk.

* All time limits indicate number of days postinoculation.

tive for rabies. Opossum 9 died on PI day 54. Mouse inoculation tests and histopathologic examination of the brain were negative for rabies. Opossums 15 and 16 were each inoculated intracerebrally with 0.3 ml. of a 20 per cent suspension of brain from opossum 9. Opossum 15 was killed on PI day 225. Opossum 16 died on PI day 84. Mouse-inoculation tests of brain and salivary gland of both animals were negative for rabies.

The cause of death in opossums 2, 9, 11, and 16 was not determined by necropsy, bacteriologic examination, mouse inoculation, or histopathologic examination. These observations seemed to indicate that the opossums used in the experiment had a high degree of resistance to rabies virus.

If deaths were due to rabies virus, it would seem that passage through the opossum had altered the virulence of the virus for mice. In no instance was rabies virus recovered from these opossums by mouse intracerebral inoculation.

Experiment 2.—To determine whether this apparent resistance was due to a peculiar host-strain relationship, the strain of virus isolated from a fox was used for further trials.

Eleven opossums were used in this experiment. By using large infective doses, it was hoped to increase the probability that clinical rabies would develop in these opossums.

Dilutions of a suspension of brain material from the original fox were used to prepare infective inoculations. Immediately after inoculation of the opossums, the infective suspensions were assayed by mouse-inoculation tests to determine the doses given. A variety of doses and routes of administration were used (table 1).

Opossums 201, 203, 204, 207, and 214 were inoculated by way of the masseter muscles. Opossums 202, 213, and 219 were inoculated intracerebrally. Opossum 209 was inoculated through the left eye into the optic nerve. Opossum 218 was inoculated into the submucosa of the nasal septum and nasal turbinates by multiple needle puncture. Opossum 210 was not inoculated but was confined in the same cage as opossum 218. Opossums 203, 210, 213, 218, and 219 died as a result of trauma sustained during blood collection by cardiac puncture on PI day 45. Opossums 207 and 214 died of salmonellosis at PI days 29 and 45, respectively. Salmonella infection was probably present when the animals were captured. Opossum 201 died of pneumonia on PI day 66. The 3 remaining opossums 202, 204, and 209 remained healthy until they were killed at PI days 55, 83, and 79, respectively.

In the entire series of 11 opossums, histologic examinations of brains and mouse-inoculation tests with brain and salivary gland suspensions all proved negative for rabies.

Experiment 3.—The CVS strain of fixed rabies virus of mouse origin was used. The $l.d._{50}$ titer for intracerebrally inoculated mice 18 to 21 days old was $10^{-6.9}$. Six opossums were inoculated into the masseter muscles with doses ranging from 1,175 to 1,175,000 mouse $l.d._{50}$ units (table 2). Opossums

TABLE 1—Method of Inoculation of Opossums and Cause of Death—Experiment 2

Opossum (No.)	Inoculation: Mouse $l.d._{50}$ units	Inoculation: Route	Survival postinoculation (days)	Cause of death
201	666,667		66	Pneumonia
203	218,730	Intramuscular into masseter muscles	46	Trauma
204	333,333		83	Killed
207	109,365		45	Salmonellosis
214	333,333		29	Salmonellosis
202	54,682		55	Killed
213	109,365	Intracerebral	45	Trauma
219	54,682		45	Trauma
209	54,682	Optic nerve	79	Killed
218	54,682	Nasal mucosa and turbinates	45	Trauma
210	None	Exposed to opossum 218	45	Trauma

33 and 31 died on PI days 62 and 105, respectively. The 4 remaining were killed on PI day 369.

No histologic examination of these opossum brains was made. Mouse-inoculation tests were carried out, using brains and salivary glands of each of the 6 opossums. Results were negative for the presence of rabies virus.

Experiment 4.—The CVS strain of rabies virus was inoculated intracranially into opossum 51 and opossum 53. They received 21,500 and 17,200 mouse $l.d._{50}$ units, respectively.

Opossum 53 failed to show signs of disease and was killed after 221 days. Brain and salivary gland tissues were used for mouse-inoculation tests and proved negative for rabies virus.

Opossum 51 showed signs of ascending paralysis which began on PI day 34. At first the right hindlimb was affected, then both hindlimbs, then the right forelimb. The opossum refused to eat and drink, and saliva drooled from its mouth. Saliva swabs taken on PI day 42 were used for mouse-inoculation tests. These tests were negative for rabies virus. Healthy opossums normally drool saliva when disturbed, and the quantity observed may not have been abnormally large. At PI day 43, opossum 51 was *in extremis* and was killed. Brain and salivary gland tissues were used in mouse-inoculation tests. The salivary gland tissue was found to be negative for rabies virus. The brain tissue contained a transmissible agent which was lethal for mice and was transmitted successfully for three passages in mice. No Negri bodies were

TABLE 2—Survival of Opossums Inoculated Intramuscularly with CVS Strain of Rabies Virus—Experiment 3

Opossum	Mouse $l.d._{50}$ units	Survival postinoculation (days)	Cause of death
31	1,175,000	105	Pneumonia
32	587,500	369	Killed
33	235,000	62	Killed
35	23,500	369	Killed
36	11,750	369	Killed
39	1,175	369	Killed

found in the brains of these mice. However, the CVS strain of rabies virus inoculated into opossum 51 does not usually produce typical Negri bodies.

SUMMARY

Of 34 opossums inoculated with varying quantities of three different strains of rabies virus by a variety of routes, only 4 showed signs of disease affecting the central nervous system and a transmissible agent lethal for mice was recovered from only 1.

The negative results obtained after large doses of virus had been inoculated intramuscularly into 3 opossums suggest that the opossum is highly resistant to rabies.

In the four experiments described, 8 opossums were inoculated intracerebrally with rabies virus. Two of these developed a disease with central nervous involvement even though the amount of virus inoculated was considerably less than that administered by other routes into many others which survived. This disease was not identified. However, it is possible that it represents atypical rabies in the opossum. If so, intracerebral inoculation may be a more reliable method of infecting the opossum. Even by this route, infection would appear to be inconsistent or erratic. Further studies will be required to determine the most reliable method of causing infection, if indeed rabies can be reproduced in the opossum.

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A Comparison of Nematode Egg Counting Techniques for Cattle and Sheep Feces

NORMAN D. LEVINE, Ph.D.; KRISHNA N. MEHRA, B.S., M.Sc., M.S.;
DAVID T. CLARK, Ph.D.; I. JOHN AVES, B.S., D.V.M.

Urbana, Illinois

SEVERAL different techniques are in current use for counting nematode eggs in feces, and each has many modifications. The three most used in the veterinary medical field are the Stoll dilution technique,[12] the McMaster technique,[3, 15] and the direct centrifugal flotation (DCF) technique which was introduced by Lane [7, 8] and modified by Stoll.[13]

Since few comparisons have been made of these different methods, it is difficult to know whether a count reported by one worker represents the same actual number of eggs per gram of feces (e.p.g.) as a count reported by another.

Stoll [12] compared his modification of the DCF technique with his dilution count technique on 43 sheep fecal samples containing, primarily, *Haemonchus contortus* eggs and found that the DCF technique was 90 per cent as efficient as the dilution count. Stoll and Hausheer [14] found that the DCF technique was 81 per cent as efficient as the dilution count for *Necator americanus* eggs. Hausheer, Herrick, and Pearse [4] found that it was 68 per cent as efficient as the dilution count for human hookworm eggs. Stoll [12] thought that the eggs of Bunostomum and Nematodirus would be demonstrable with greater facility than those of Haemonchus, but that those of the smaller strongyles would be less so. He stated, "So far as our observations go, the ova of such worms as the various species and class relatives of Ascaris and Trichuris do not come off well in DCF preparations, although they are readily counted by dilution."

Wilson [16] reported no significant difference in egg counts made by the DCF and McMaster techniques on 130 fecal samples from lambs experimentally infected with *H. contortus*, *Trichostrongylus axei*, and *Ostertagia circumcincta*.

Peters and Leiper [10] found that the McMaster technique gave significantly higher counts of sheep nematode eggs than the Stoll dilution method. On the basis of the evidence available, however, they could not show that it was significantly more accurate. They felt that there was some evidence of overestimation by the McMaster technique and of underestimation by the Stoll.

Farr and Luttermoser [1] found that the DCF technique resulted in recovery of 15 per cent of the *Ascaridia galli* and *Heterakis gallinae* eggs and 10 per cent of the mixed coccidian oocysts present in chicken feces when zinc sulfate (331.4 Gm. in 1,000 ml. solution) was used as the flotation fluid, and 26 per cent of the *A. galli* and *H. gallinae* eggs and 6 per cent of the coccidian oocysts when Sheather's sugar solution was used.

MATERIALS AND METHODS

So many different modifications—some of them unconsciously so—of the basic counting techniques have been used that it is important to know exactly how the counts were made in order to be able to compare them properly. Even what might be thought to be minor variations may affect the results. Stoll [13] gave an excellent discussion of this problem.

The McMaster technique which we used was a slight modification of that described by Whitlock.[16] Two grams of feces were placed in a shell vial and 28 ml. of water added. If the feces were in the form of pellets, they were crushed as well as possible, set aside for an hour or more and then finely broken up. The suspension was then mixed thoroughly; 1 ml. was placed in a test tube, 1 ml. of Sheather's sugar solution was added, and the suspension was again mixed thoroughly. While it was still being kept in motion, some of it was withdrawn with a pipette and placed quickly in a McMaster counting chamber, making sure that the marked area in the chamber was filled. The preparation was allowed to stand for a few minutes to allow the eggs to come to the top. Then the chamber was placed on the microscope stage and the eggs in the marked area were counted. The numbers of eggs in two to four chambers (usually the latter) were determined, and each count was recorded as the average of these numbers.

Both commercially purchased and homemade McMaster counting slides were used. In the former, the chamber was 1.5 mm. high and the multiplication factor to convert the count to numbers of e.p.g. was 300, while in the latter the chamber was 1.0 mm. high and the multiplication factor was 200.

The DCF technique which we used was essentially that described by Stoll [13] except that different flotation solutions were used. Ten grams of feces was placed in a 300-ml. Erlenmeyer flask; 290 ml. of water was added and mixed thoroughly; 15 ml. of the suspension was poured quickly into a centri-

From the College of Veterinary Medicine and the Agricultural Experiment Station, University of Illinois, Urbana; Dr. Clark is now at the College of Veterinary Medicine, Michigan State University, East Lansing, and Dr. Aves is in Belvidere, Ill.

fuge tube and centrifuged for five minutes in an International No. 1 centrifuge at approximately 2,000 r.p.m. (Stoll centrifuged for 1 minute at 1,000 r.p.m.). After the centrifuge had been allowed to come to a stop without braking, the supernatant fluid was poured off. A small amount of the flotation solution was added and mixed with the sediment at the bottom of the tube. The tube was then filled to the top with the flotation solution so that the meniscus rose slightly above the top of the tube. A round coverslip was placed on top of the tube, and the tube was centrifuged as before. After the centrifuge had come to a stop, the coverslip was lifted off cleanly and placed on one end of a slide. It was not allowed to slide over the edge of the tube. Carelessness in picking off the coverslip will markedly reduce egg recovery.

An amount of flotation solution equal to that removed by the coverslip was added to the centrifuge tube, another round coverslip was placed on it, allowed to stand five minutes, picked off and placed on the other end of the slide. All the eggs on the two coverslips were counted and the total number multiplied by two to obtain the total number of e.p.g.

In one series of 26 cattle fecal samples and in some other cases, the eggs on three successive coverslips were counted in order to determine the percentage of eggs recovered on each one.

Direct egg counts were made on fecal samples from 13 cattle by mixing a little physiologic sodium chloride solution with 0.1 Gm. samples of feces on a slide and counting every egg present. This procedure was tedious, taking at least 3 hours per sample, but was felt desirable in order to give a solid foundation for comparisons.

The Sheather's sugar solution used in this study was prepared by dissolving 500 Gm. of sucrose and 6.5 ml. melted phenol in 360 ml. distilled water. The zinc sulfate solution was prepared by dissolving 331 Gm. $ZnSO_4 \cdot 7H_2O$ in 1,000 ml. of water. The saturated zinc sulfate and sodium nitrate solutions were made by adding excesses of the salts to distilled water, heating, and allowing the solutions to cool.

Only eggs of nematodes of the suborder Strongylorina were counted in this study. This group includes the genera Haemonchus, Trichostrongylus, Cooperia, Ostertagia, Oesophagostomum, Chabertia, and Bunostomum. Some Strongyloides, Trichuris, and Nematodirus eggs were seen, but these were too few for valid comparisons, and they are not included.

Student's "t" test was used for statistical analyses.

RESULTS

When the coverslip is picked off the tube in the DCF technique, some eggs usually fail to adhere to it. These can be recovered by using one or more additional coverslips as described above. In order to determine how many coverslips should be used to obtain all, or practically all, the recoverable eggs, the numbers of eggs recovered from three successive coverslips were determined in flotations from a series of 26 cattle. Duplicate flotations were made on each fecal sample with both Sheather's sugar and saturated sodium nitrate solutions. The results are summarized (table 1); 98 and 96 per cent, respectively, of the eggs were recovered on the first two coverslips.

TABLE 1—Numbers of Cattle Strongyle Eggs on Successive Coverslips on Direct Centrifugal Flotation (Duplicate Samples from 26 Cattle Examined)

	Flotation solution	
	Sat. sodium nitrate	Sheather's sugar
Total number of eggs observed	1,721	2,377
Percentage of eggs observed:	.....	.....
On first coverslip	79	88
On second coverslip	17	10
On third coverslip	4	2

The results of the egg counts obtained by the different techniques are summarized (table 2). Comparisons were made on one series of fecal samples from 13 sheep and on four series of samples from 15, 24, 26, and 13 cattle, respectively. All counts in each series were made by the same person. The differences between the results obtained with the different flotation solutions by the DCF technique in series 3, 4, and 5 were not significant at the 5 per cent level, nor was the difference between the results obtained by the McMaster and direct count techniques in series 5. On the other hand, the differences between the results obtained by the McMaster and DCF techniques in all five series were significant far beyond the 0.1 per cent level.

DISCUSSION

The above data show that the counts obtained by the McMaster technique were accurate indexes of the numbers of eggs actually present, while the counts obtained by the DCF technique were too low. The Stoll dilution technique is also generally recognized as accurate.[10] However, since the McMaster technique is much faster (and, indeed, faster than the DCF technique also), it is preferable. The sole advantage of the DCF technique over the McMaster technique is that it may be positive when the number of eggs present in the sample is too small to be counted by dilution meth-

ods. Stoll [13] recommended the DCF technique for counts of less than 400 e.p.g. However, in a careful statistical examination of worm egg count sampling technique for sheep, Hunter and Quenouille [5] showed that the efficiency of the count was 80 per cent if the mean number of eggs in the McMaster chamber was four, and 50 per cent if it was one. With dilution factors of 200 and 300, obtained in chambers 1.0 and 1.5 mm. deep, respectively, this means that if the eggs in four chambers are counted, the efficiency of counts of 200 and 300 e.p.g., respectively, would be 80 per cent, and that of counts of 50 and 75 e.p.g., respectively, would be 50 per cent.

A number of comparisons have been made of different flotation fluids for the DCF technique. In all but a few of these, no actual egg counts were made, comparisons being based on the percentages of known positive cases revealed by the different solutions. These studies are not pertinent to the present one. However, in a comparison of the efficiency of saturated sodium nitrate (sp. g., 1.400), saturated zinc sulfate (sp. g., 1.380), 62 per cent magnesium sulfate (sp. g., 1.285), saturated sodium chloride (sp. g., 1.200) and Sheather's sugar (sp. g., 1.275) solutions for flotation of helminth ova and coccidian oocysts in dog feces, Koutz [6] gave average egg and oocyst counts. He did not give individual counts or analyze his data statistically, so it is impossible to be sure whether many of the differences he observed were significant. In addition, he centrifuged his samples for only two minutes. He obtained the highest egg counts with sodium nitrate, but because of the amount of debris and large number of air bubbles obtained with it, he considered that zinc sulfate, sugar, and magnesium sulfate were of greater diagnostic value. On the basis of his data, we calculated that 60 per cent as many *Trichuris vulpis* eggs, 92 per cent as many *Toxocara canis* eggs, 82 per cent as many *Toxascaris leonina* eggs, 47 per cent as many *Ancylostoma caninum* eggs, 55 per cent as many *Troglotrema salmincola* eggs, 13 per cent as many *Isospora bigemina* oocysts, and 36 per cent as many *Isospora rivolta* oocysts were obtained with Sheather's sugar solution as with sodium nitrate. The comparable percentages for zinc sulfate solution were 20, 75, 84, 39, 103, 54, and 57 per cent, respectively; those for magnesium sulfate were 12, 45, 46, 41, 96, 4, and 61 per cent, respectively; and those for sodium chloride were 0.2, 38, 45, 31, 0.0, 27, and 26 per cent, respectively.

Ray [11] compared zinc sulfate, sugar, and sodium chloride solutions, all of sp. g. 1.200, for the flotation of sheep and goat coccidian oocysts. He thought that sugar and sodium chloride were better than zinc sulfate, but our statistical analysis of his data revealed that there was no significant difference between the three. Gill [2] found no statistically significant difference between copper nitrate, sodium chloride, and zinc sulfate for the flotation of chicken coccidian oocysts.

In our own work, we found no significant difference between Sheather's sugar solution and saturated sodium nitrate solution or between 331 Gm. zinc sulfate per liter of water and a saturated zinc sulfate solution (approx. 965 Gm. per liter of water). There are other differences, however, which make some flotation fluids preferable to others. We prefer Sheather's sugar solution for nematode eggs because it does not crystallize as readily as salt solutions, causes less distortion than either sodium chloride or

TABLE 2—Comparison of Strongyle Nematode Egg Counting Techniques for Cattle and Sheep Feces

Series (No.)	Species and number of animals	Mean number of eggs per gram feces found by McMaster technique [1]	Mean number of eggs found as percentage of the number found by the McMaster technique				
			Direct count	Direct centrifugal flotation technique			
				Sheather's sugar soln.	331 Gm./liter zinc sulfate	Sat. zinc sulfate	Sat. sodium nitrate
1	13 Sheep	285	....	11	....	....	....
2	15 Cattle	78	....	22	....	....	....
3	24 Cattle	135	....	....	22	27	....
4	26 Cattle [2]	351	....	46	....	....	36
5	13 Cattle [3]	783	92	51	....	....	56

[1] Flotation fluid, Sheather's sugar solution. [2] Two counts made on each sample by each technique. [3] Two counts made on each sample by McMaster and direct centrifugal flotation techniques; all eggs in 0.1 Gm. of feces counted in the direct count.

sodium nitrate, and has fewer debris or air bubbles than sodium nitrate. However, for protozoan cysts other than those of coccidia, zinc sulfate is preferable.

Several factors affect the accuracy of the DCF technique. As mentioned in the description of the technique, the way in which the coverslip is removed from the centrifuge tube affects the number of eggs which adhere to it. Stoll [13] found that when the coverslip was picked off carefully, about 98 per cent of sheep Haemonchus eggs were present on the first two coverslips, 87 per cent being on the first and 11 per cent on the second. Ray [11] found that the first coverslip floated an average of 84 per cent of the sheep and goat oocysts recovered with sugar solution, 79 per cent with sodium chloride, and 85 per cent with zinc sulfate. Gill [2] found that the first coverslip floated 84 per cent of the chicken coccidian oocysts recovered with copper nitrate solution, 93 per cent with sodium chloride, and 91 per cent with zinc sulfate. These are comparable to our own finding that 88 per cent of the cattle strongyle eggs recovered were found on the first coverslip and 10 per cent on the second with Sheather's sugar solution and 79 per cent and 17 per cent, respectively, with sodium nitrate.

The duration and speed of centrifugation can be expected to affect the number of eggs recovered. If these are not adequate with respect to each other, a certain proportion of the eggs will not reach the top.

Another possible but unproved source of variation in the DCF technique is what might be called the personal factor. Minor differences in technique between different operators, even when they are all following the same directions, might affect the numbers of eggs recovered. So far as we are aware, no direct comparisons have been made to discover whether such differences exist, but they might account in part for differences in efficiency reported by different operators.

One question worth mentioning is what happens to the eggs which the DCF technique fails to recover. Examination of the sediment in the centrifuge tubes showed that eggs are not there in significant numbers. They must, therefore, be still suspended in the flotation fluid.

In view of the above considerations, it is clear that comparisons of egg counts made by different operators with any but the McMaster and Stoll techniques would be subject to serious error. This fact is of major importance if one wishes to use egg counts to help evaluate the severity of parasite infections. For example, Levine and Aves [9] considered that 300 mixed strongyle e.p.g. was suggestive of borderline or subclinical infection in cattle. This estimate was based on counts made by the McMaster technique. Comparable counts by the DCF technique would have ranged from 66 to 168 e.p.g. in the present study. For valid comparisons of results from one laboratory to another, a standard egg-counting technique must be used. Because of its speed and accuracy, the McMaster technique is best for this purpose.

SUMMARY

In a comparative study of fecal samples from 78 cattle and 13 sheep, the McMaster dilution technique was found to be far superior to the direct centrifugal flotation (DCF) technique for counting strongyle nematode eggs. In a series of 13 sheep, the average egg count obtained by the DCF technique with Sheather's sugar solution as the flotation fluid was 11 per cent of that obtained by the McMaster technique. In a series of 15 cattle, the average egg count obtained by the DCF technique with Sheather's sugar solution was 22 per cent of that obtained by the McMaster technique. In a second series of 24 cattle, the average egg counts obtained by the DCF technique with 33.1 per cent and saturated zinc sulfate solutions were 22 and 27 per cent, respectively, of those obtained by the McMaster technique. In a third series of 26 cattle, the average egg counts obtained by the DCF technique with Sheather's sugar solution and sodium nitrate solutions were 46 and 36 per cent, respectively, of those obtained by the McMaster technique. In a fourth series of 13 cattle, the average egg counts obtained by the DCF technique with Sheather's sugar and saturated sodium nitrate solutions were 51 and 56 per cent, respectively, of those obtained by the McMaster technique, while direct egg counts made by counting every egg in 0.1 Gm. fecal samples averaged 92 per cent of those obtained by the McMaster technique. The differences between the results obtained with different flotation solutions by the DCF technique were not significant at the 5 per cent level, nor was the difference between the results obtained by the McMaster and direct count

techniques. The differences between the results obtained by the McMaster and DCF techniques were significant far beyond the 0.1 per cent level.

It is concluded that for valid comparisons of fecal egg counts to be made between different laboratories, a standard counting technique must be used. Because of its speed and accuracy, the McMaster technique is best for this purpose for cattle and sheep feces, while the DCF technique is not.

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Scientific Notes

Urea Clearance as Measured by Titration with Mercuric Chloride

M. J. BURNS, Ph.D.

OF ALL SUBSTANCES in the urine, urea has been of greatest interest to both the physiologist and the clinician. It is the chief nitrogenous end-product of protein metabolism and, except for water, the chief constituent of urine.

Blood urea clearance represents the number of milliliters of blood cleared of its urea content in one minute's excretion of urine. In its application, accuracy in blood and urine analyses and in urine collection are of paramount importance.

The urea clearance is more sensitive than the level of blood urea alone as an evaluation of the renal status. Values below 70 per cent of the average normal clearance imply impairment of renal function, even though the blood urea concentration may be within normal limits. Values between 25 and 50 per cent of normal are usually associated with a rise in the concentration of blood urea. With still lower clearance, the blood urea levels increase rapidly. Ralli et al.[7] found that the urea clearance in dogs varied from 76.5 to 121.4 ml. per square meter of surface area; Summerville et al.[10] found variations from 62.2 to 99.6 ml. per square meter of body surface.

There are numerous reports in the literature concerning urea clearance in the dog[1, 2, 5–11]; however, none of these made reference to the mercuric chloride method for the determination of urea in urine. This method [8] has been in common use for the determination of urea in blood and, in the hands of an inexperienced technician, is more accurate than urease methods, since it offers less chance of error in technique. Only simple reagents and inexpensive equipment are required for its use.

The object of this experiment was to devise a method for assay of urea in urine based on the mercuric chloride method and to follow the urea clearance in dogs in which renal damage had been produced by uranyl nitrate hexahydrate.

MATERIALS AND METHODS

Procedure for Urine Urea—One milliliter of urine is diluted to 25 ml. with distilled water. To prepare a test sample, 2.5 ml. of 10 per cent trichloroacetic acid is added to 2.5 ml. of diluted urine in a 50-ml. beaker. (It is not necessary to filter or centrifuge the test sample as is required for blood.)

To titrate the test sample, add about 2 ml. of 5 per cent mercuric chloride from a burette and then test a drop of the mixture with a drop of saturated sodium carbonate on a porcelain spot plate. Titration is continued by adding mercuric chloride until a test drop of the mixture produces a reddish-brown precipitate which appears within three seconds. It is important to mix the mercuric chloride and diluted urine thoroughly before the test drop is removed. A yellow precipitate will occur before the reddish-brown precipitate, but titration is continued until the precipitate turns reddish-brown within three seconds.

To calculate the content of urea in urine, subtract 1.5 ml. from the total amount of mercuric chloride used and multiply the difference by 100 to obtain the number of milligrams of urea per milliliter of urine. (1.5 ml. of mercuric chloride combines with nonprotein nitrogen compounds other than urea.) Multiply the result by 46.6 per cent to obtain urine urea nitrogen values.

Example:

4.5 ml. total mercuric chloride used
−1.5

3.0 ml. × 100 = 300 mg. urea/ml. of urine

300 × 46.6 = 139.8 mg. urine urea N/ml. of urine

A urease method was used to determine urine urea as a means of comparison[4]; reagents used for this method are: isotonic sodium sulfate solution (13.2 Gm. anhydrous sodium sulfate per liter of solution); zinc sulfate solution (10 Gm. $ZnSO_4 \cdot 7H_2O$ per 100 ml. of solution); 0.5 N NaOH; Nessler's reagent; and standard urea soultion. For the standard urea solution, dry about 0.5 Gm. of pure urea at 100 C. for one hour and cool in desiccator. Weigh accurately 100 mg. and dissolve in water to a total volume of 100 ml. The solution is preserved by means of a drop of chloroform and kept in the cold.

Using these reagents, the following ingredients

From the Department of Physiology, School of Veterinary Medicine, Auburn University, Auburn, Ala., where Dr. Burns is associate professor. Approved by the Committee on Publications as paper No. 846.

are measured accurately into a series of clean, dry, test tubes:

	Test *	50-mg. std.	100-mg. std.	Blank
Isotonic sodium sulfate solution	3.3 ml.	3.3 ml.	3.2 ml.	3.4 ml.
Diluted urine (1:25 dilution)	0.1 ml.	.....	.....	.....
Standard urea solution	.....	0.1 ml.	0.2 ml.	.....

* For blood urea—use 3.2 ml. sodium sulfate and 0.2 ml. whole blood.

A "knife point" (about 20 mg.) of Jack-Bean meal is added to each tube. The tubes are stoppered and the contents are mixed well. After incubation at 37 C. for 20 minutes, 0.3 ml. of zinc sulfate solution and 0.3 ml. of 0.5 N sodium hydroxide are added to each tube. The contents are mixed well after each addition and then centrifuged at high speed for two to three minutes.

The spectrophotometer is set at 480 mμ and adjusted to 100 per cent transmission using distilled water.

Five milliliters of distilled water and 2.0 ml. of the supernatant fluid from each tube are placed in each of a series of cuvettes. The contents of the tubes are mixed by shaking, and then 1.0 ml. of Nessler's reagent is added quickly to one cuvette at a time. Optical density of the contents of each cuvette is read immediately after the addition of the Nessler's reagent. The reading of the blank D (O) is noted and subtracted from the readings of the test D (T) and the 50- and 100-mg. standards D (50) and D (100).

The following formula is used to calculate the urea concentrations:

$$\text{mg. urea/ml. urine} = \frac{D(T) - D(O)}{D(50) - D(O)} \times 25$$

$$\text{or } \frac{D(T) - D(O)}{D(100) - D(O)} \times 50$$

Procedure for Production of Renal Damage and Collection of Urine—Using 2 dogs of mixed breeding, each weighing 10 kg., a 1 per cent solution of uranyl nitrate hexahydrate was injected intravenously at the rate of 0.5 ml. per kilogram of body weight (5 mg. uranyl nitrate hexahydrate per kg. body weight). The dogs were placed in metabolism cages and furnished feed and water ad libitum.

The urine of each dog was collected in a large glass container. One milliliter of formalin was added to the container at the beginning of each collection period, which varied from 18 to 24 hours. The volume of urine was measured at the end of the collection period and its urea content was determined as described.

Procedure for the Determination of Urea Clearance—The blood urea is determined on a sample of blood as follows: 5.0 ml. of 10 per cent trichloroacetic acid is placed in a 50-ml. beaker and 5.0 ml. of blood containing an anticoagulant is slowly added while stirring. The mixture is allowed to stand at least five minutes before centrifuging or filtering to obtain a protein-free filtrate. Five milliliters of the filtrate is titrated with mercuric chloride as described for the diluted urine.

To calculate the blood urea concentration, subtract 1.5 ml. from the total used and multiply the difference by 40 to obtain blood urea concentration per 100 ml. of blood.

To calculate blood urea clearance, the following formula is used:

$$\text{Urea clearance} = \frac{\text{Quantity of urine (ml./min.)} \times \text{Urea in urine (mg./ml.)}}{\text{urea in blood (mg./ml.)}}$$

RESULTS

When the mercuric chloride method was compared with the urease method for the determination of urine urea, the average of ten samples for each method was 29.5 and 27.0 per cent, respectively.

The relationship between blood urea nitrogen and urea clearance in both of the dogs is shown (fig. 1). The urea clearance decreased considerably before there was a significant increase in the blood urea concentration. One week after the uranyl nitrate had been administered, the blood urea concentration was normal but the urea clear-

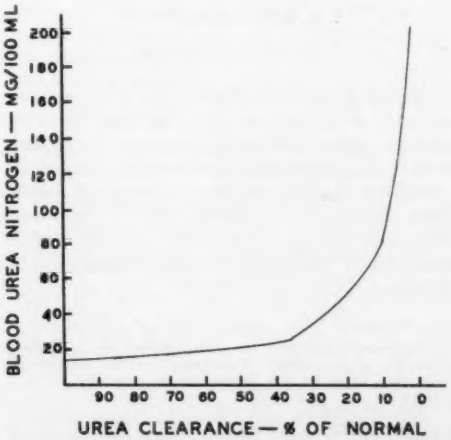

Fig. 1—Relationship of urea clearance to blood urea nitrogen concentration (average data for 2 dogs).

ance had dropped to approximately 46 per cent of normal. Ten days after the administration of the uranyl nitrate, the blood urea nitrogen had risen to about 75 mg. per 100 cc. of blood and the urea clearance had dropped to 14 per cent of normal. At the end of the second week, the blood urea nitrogen had risen to 186 mg. and the urea clearance was only 2 per cent of normal. The dogs died on the fifteenth day.

The normal urea clearance in the 2 dogs studied was approximately 65 ml. per square meter of body surface. The following formula was used to calculate body surface:

$$\text{Surface area (sq.M)} = \frac{\text{Body weight (Gm.)}^{0.66}}{1{,}000}$$

SUMMARY

A simplified method is described for the determination of urea clearance. Mercuric chloride is used to titrate both the blood urea and urine urea. The comparison of the urine urea method and a urease method is presented.

The method was used to follow the renal function of dogs given intravenous injections of uranyl nitrate. The results of these tests show that the urea clearance decreases significantly before there is a significant decrease in the blood urea concentration and that urea clearance is a more sensitive test for kidney function than blood urea values alone.

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A Catheter for Nasal Inoculation of Large Animals

E. R. DOLL, M.A., D.V.M.

Lexington, Kentucky

A FLEXIBLE plastic catheter * has been designed for introducing inoculums into the nasal passages and nasopharynx of large animals. It was used initially for transmission experiments in the investigation of viral respiratory diseases of horses. More recently, it has been used for intranasal administration of a live virus vaccine that is being tested for control of viral rhinopneumonitis of horses.

The device (fig. 1) is 12 inches long. The diameter of the body is 8 mm. The tip is tapered to provide a portion 1 inch long and 4 mm. in diameter. Five capillary pores are provided in the

Fig. 1—A nasal inoculation catheter for large animals. Wires have been inserted in the capillary pores to show their position.

From the Department of Animal Pathology, University of Kentucky Agricultural Experiment Station, Lexington. Published with the permission of the director of the station.

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* Available from Goshen Laboratories, Inc., Goshen, N.Y.

tip. One pore discharges fluid forward from the tip and the other four pores discharge fluid laterally at 90-degree angles. The bore of the tube is slightly less than the diameter of a 16-gauge needle. In use, the butt of the catheter is slipped over a 16-gauge needle which may be fitted to any type of syringe equipment. For field use, it is attached to syringes with automatic filling devices.

This device would be useful to research workers who are investigating respiratory diseases of cattle. It would also be useful for introducing inoculums into the nasal passages of sheep and swine of reasonable size and for applying liquid medicament to the nasal passages and nasopharynx. By moving the catheter back and forth and by rotating it, an inoculum or medicament may be applied to a large area of the nasal mucosa.

An Improved Training Aid: Preservation of Meat for Instruction of Veterinarians

MARION A. WILCOX

Washington, D.C.

AN IMPROVED visual training aid has been developed at the Medical Museum Laboratory of the Armed Forces Institute of Pathology. It was specifically designed to aid in the instruction of meat inspectors. The instruction program requires the frequent use of samples of meat such as pork chops and bacon. The preservation and mounting of various types of meats have made available to students and teachers a stock of training material that previously had to be purchased fresh for each new class. Attractively mounted meat specimens have proved both useful and economical. The time as well as the money required for obtaining new material for each new class is reduced.

The value of this method is that true color in certain meats, an important factor in training students to recognize the difference between normal and diseased meats, is preserved. Preservation and color restoration in meats were accomplished through modifying and combining the Klotz-Kaiserling-Shein museum fluids.*

Fig. 1—Section of preserved pork chop.

MATERIALS AND METHODS

Specimens were immersed in the following fixative for one week or more:

Sodium chloride	28 Gm.
Sodium bicarbonate	16 Gm.
Chloral hydrate	16 Gm.
Formaldehyde (40 per cent)	200 ml.
Water, distilled q.s.	4,000 ml.

A fresh surface was cut and the specimen coated with the following gelatin solution:

Gelatin, granulated, U.S.P.	115 Gm.
d-sorbite (sorbitol)	10 Gm.
Methyl violet dye (0.5 per cent)	2 or 3 drops (optional)
Water, q.s.	1,000 ml.
Formaldehyde (40 per cent)	5 ml./100 of gelatin solution

In preparing the coating, care should be exercised to keep the temperature of the gelatin below 60 C. The formaldehyde, which hastens the setting of the coating, is added to the gelatin solution when the technician is ready to coat the specimen.

From the Medical Museum, Armed Forces Institute of Pathology, Washington, D.C.

* Preserving fluids for gross specimens. In "Manual of Macropathological Techniques." Armed Forces Institute of Pathology, Medical Museum, Washington, D.C., (1957): 6-7.

Use the following solution when ready to mount the specimen in a plastic container:

Sodium phosphate monobasic	18.76 Gm.
Sodium phosphate dibasic	23.68 Gm.
Water, distilled q.s.	4,000 ml.
Formaldehyde (40 per cent)	200 ml.
Sodium hydrosulfite	20 Gm.

SUMMARY

A method of preserving and retaining color in certain meats for exhibit and study by veterinarians has been achieved through the modifying and combining of Klotz-Kaiserling-Shein solutions. The method saves time and reduces cost when the same type of material must be used regularly.

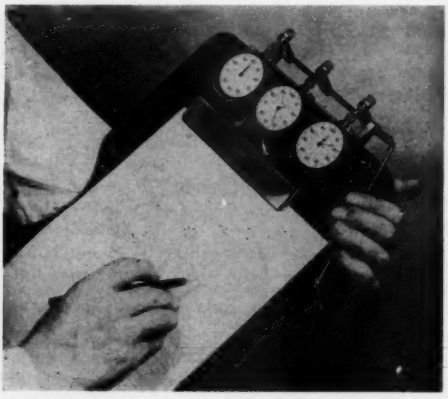
Research Tools

Further information on these products may be obtained by writing to: Research Tools, American Veterinary Medical Association, 600 S. Michigan Ave., Chicago 5, Ill.

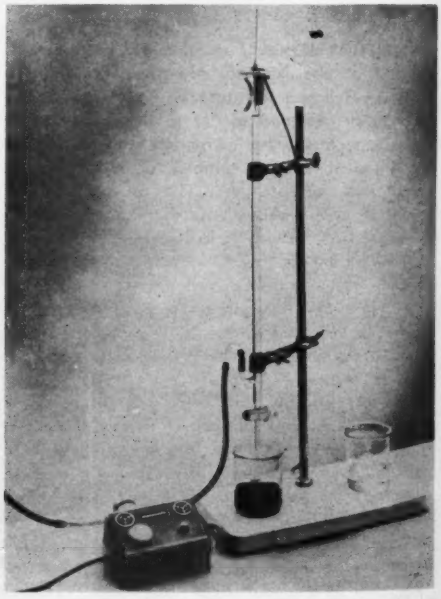
Sequence Timing Device

A clip board with a device for actuating three stopwatches at the same instant is available from the Heuer Timer Corporation.

A squeeze of the hand which holds the board operates the device. Sequential steps are timed with zero time loss between steps, and it is not necessary to read the timer hands while they are in motion. Timing is started with the first watch at zero, the second watch stopped at any point other than zero, and the third watch in motion. Each step is timed in with the same motion that the preceding step is timed out. With central-register timers data are recorded in hundredths of a minute for simplified per minute rate calculations. Hour-decimal timers and standard fifth-of-a-second timers can also be used. Watches may be wound and operated without removal from the board.



Automatic Burette Filler

Increased speed and accuracy in titrations can be obtained with the automatic burette filler now available from Coleman Instruments, Inc. The elimination of manual replenishment and adjustment of burette contents to volumetric zero for each titration reduces titration time by up to 30 per cent.

The instrument automatically refills 5-, 10-, 25-, or 50-ml. open-top, side-filling burettes. An electrically actuated valve is connected into the supply line between the reservoir and the burette. A push-button control opens this valve, allowing the material to flow into the burette. The valve automatically closes when the material reaches a sensing probe, which is clamped to the top of the burette and adjusted to the volumetric zero mark. Any liquid that is sufficiently ionized to be electrically conductive can be used with this device.